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The Role of galectin-3 in liver progenitor cell proliferation and differentiation

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ABSTRACT

Liver progenitor cells (LPCs) respond to hepatic injury when hepatocyte division is impaired in chronic or severe injury. The LPCs are intimately surrounded by myofibroblasts, macrophages and laminin, thus constituting a potential progenitor cell niche. Laminin has been proposed to maintain LPCs in an undifferentiated state within the LPC niche. LPCs differentiate once they leave the laminin niche. However, mechanisms regulating this process have not been completely investigated. I hypothesized that cell membrane proteins which are implicated in intergin activation and mediation of cell adhesion to laminin such as galectin-3 and CD98 may be involved in this mechanism. Galectin-3 is a carbohydrate-binding protein which plays an important role in various cell functions, including cell growth, proliferation, adhesion, and differentiation. Galectin-3 has been reported to bind integrins and regulates β1 mediated adhesion to ECM. In addition, galectin-3 may also indirectly mediate β1 integrin activation by binding to and activating the heterodimeric transmembrane amino acid transporter CD98. However a role for galectin-3 in regulating LPC behavior has not been demonstrated.

In this thesis, the mechanisms of galectin-3 mediating LPC proliferation and differentiation were investigated in an experimental model of LPC induction, the CDE diet, by using mutant mice lacking the gene encoding galectin-3. I have found galectin-3 is important for LPC induction and proliferation *in vivo*. In addition, galectin-3 is crucial for the LPC proliferation but is a negative regulator of LPC differentiation *in vitro* in a laminin dependent manner, suggesting that galectin-3 is required for LPC to maintain in an undifferentiated state on laminin. Moreover, the

extracellular binding activity of galectin-3 is important for LPC proliferation and adhesion to laminin. Furthermore, in the absence of galectin-3, LPCs down-regulate cyclin-D1 and the cyclin inhibitors p21 and p16 are elevated. Finally I suggest that integrin-β1 and CD98 are involved in regulating LPC proliferation.

There is an increasing literature examining the role of LPC niche in regulating LPC behavior. My work suggests that galectin-3 is required for the expansion of LPCs in the injured adult liver. Galectin-3 enhances LPC adhesion to laminin. Galectin-3 is a crucial factor for LPCs to maintain in an undifferentiated state on laminin. My findings not only emphasize the requirement of LPCs to interact with their extracellular environment to expand but also propose that galectin-3 is a key signalling intermediary in the LPC niche, regulating homeostatic balance between proliferation and differentiation of LPCs, thus controlling regeneration.

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DECLARATIOIN

This thesis has been written by myself and represents my own work. All the experiments described here were performed by me

AIMS & HYPOTHESIS

In this study, I hypothesize that galectin-3 can enhance LPC adhesion to laminin and plays an important role in LPC induction, proliferation, and differentiation. In addition, integrin activation and CD98 might be involved in regulating LPC proliferation. To investigate this hypothesis, I aim to examine the role of galectin-3 in LPC induction and proliferation *in vivo* by analyzing the galectin-3 null mice which were put on CDE diet, a model of LPC induction, for 12 days. In addition, I aim to isolate primary LPCs from galectin-3 null mice and knock down the expression of galectin-3 in LPC line: BMOLs to investigate the role of galectin-3 in LPC proliferation, differentiation and adhesion to laminin *in vitro*. Finally the mechanisms by which galectin-3 regulates LPC proliferation were investigated by examining the role of galectin-3 in regulating cell cycle regulators such as cyclin D1, p16, and p21. Cell-adhesion signalling pathway such as FAK/Akt/Erk pathways were also investigated.

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ABBREVIATION

AAF	2-N-acetylaminofluorene
AFP	Alpha-fetoprotein
ALT	Alanine aminotransferase
BCL2	B cell lymphoma 2
BMOL	Bipotential murine liver progenitor cells
BMP	Bone morphogenetic proteins
CCl4	Carbon tetrachloride
CDE	Choline-deficient ethionine-supplemented
CDKs	Cyclin-dependent kinases
CK	Cytokeratin
CKI	Cyclin-dependent kinase inhibitor
CRD	Carbohydrate recognition domain
CYP	Cytochrome P450
DDC	3,5-Diethoxycarbonlyl-1,4-dihydrocollidinesupplemented
DLK 1	Delta-like 1 protein
DMSO	Dimethyl sulfoxide
ECM	Extracellular matrix
ED	Embryonic day
EpCAM	Epithelial cell adhesion molecule
ERK	Extracellular signal-regulated kinase
EGF	Epidermal growth factor
FAK	Focal adhesion kinase
FGF	Fibroblast growth factors
FLSPCs	Fetal liver stem/progenitor cells
GGT	Gamma-glutamyl transpeptidase
GSK3 β	Glycogen synthase kinase 3 β
HCC	Hepatocellular carcinoma
Hex	Haematopoetically expressed homeobox factor
HGF	Hepatocyte growth factor

HNF	Hepatocyte nuclear factor
HSC	Hepatic stellate cells
IFNγ	Interferon gamma
IL-6	Interleukin -6
ILK	Integrin-linked kinase
LT β	Lymphotoxin beta
LPC	Liver progenitor cell
MAPK	Mitogen activated protein Kinase
MEK	MAPK kinase
OSM	Oncostatin M
PBS	Phosphate-buffered saline
PI3K	Phosphatidylinositol-3-kinase
PKB	Protein kinase B
Rb	Retinoblasma tumor supressor proteinc
Sca-1	Stem cell antigen-1
SHPCs	Small hepatocyte-like progenitor cells
STM	Septum transversum mesenchyme
TGF	Transforming growth factor
TNF	Tumour necrosis factor
TWEAK	TNF-like weak induction of apoptosis

CHAPTER 1

INTRODUCTION

1.2 Liver development and renewal

1.2.1 Liver development

During the embryo development, the endoderm germ layer is established during gastrulation and forms a primitive gut tube that is subdivided into foregut, midgut and hindgut regions (Figure 1.1 A). Liver arises from the foregut endoderm at embryonic day (ED) 8.5 and is engrafted by hematopoietic progenitor cells at ED9 to ED10. Only foregut endoderm is competent to develop into the liver (Fukuda-Taira, 1981; Le Douarin, 1975; Okada, 1954); therefore, the establishment of the foregut progenitors is an important step in hepatogenesis. The expression of transcription factors such as Foxa2, Gata4 – 6 and haematopoetically expressed homeobox factor (Hex) have been shown to play important roles in early foregut organogenesis (Bossard et al., 1998; Bossard et al., 2000; Gualdi et al., 1996; Lee et al., 2005). In addition, the hepatic fate is induced by fibroblast growth factors (FGF) signals from the developing heart and bone morphogenetic proteins (BMPs) from the septum transversum mesenchyme (STM) (Fukuda-Taira et al., 1981; Gualdi et al., 1996; Jung et al., 1999; Le Douarin et al., 1975; Rossi et al., 2001). Moreover, Wnt and FGF4 signaling must be inhibited to maintain foregut identity and allow hepatic induction (Dessimoz et al., 2006; McLin et al., 2007; Wells and Melton, 2000), whereas becomes necessary to promote liver bud emergence and differentiation (McLin et al., 2007) (Zorn et al., 2008).

At ED 9.0, the hepatic diverticulum, an out-pocket of thickened ventral foregut epithelium adjacent to the developing heart, was formed (Figure 1.1B). This is the first morphological sign of the embryonic liver. The anterior portion of the hepatic diverticulum gives rise to the liver and intrahepatic biliary tree, while the posterior portion forms the gall bladder and extrahepatic bile ducts. Around ED 9.5, undifferentiated endodermal cells, known as hepatoblasts proliferate and then invade the adjacent STM to form the liver bud (Houssaint et al., 1980; Le Douarin et al., 1975) (Zorn et al., 2008). A number of studies in mutant mice have shown that liver bud formation is tightly controlled by a network of transcription factors such as hex (Martinez Barbera et al.,2000; Bort et al., 2006), GATA-6 (Zhao et al., 2005), hepatocyte nuclear factor (HNF)-6 (Margagliotti et al., 2007), Onecut (OC)-2 (Margagliotti et al., 2007), and prospero-related homeobox 1 (Prox-1) (Sosa-Pineda et al., 2000)

Around ED 9.0- ED 9.5, the hepatoblasts start to express α-fetoprotein (αFP) followed by albumin then begin to express intermediate filament proteins (CK-14, CK-8, and CK-18), and γ-glutamyl transpeptidase (GGT) while proliferating (Brill et al., 1995; Tee et al., 1996) from ED 11. Hepatoblasts are bipotential and selectively differentiate into either hepatocytes or bile duct epithelial cells before ED16, which is a critical time to determine the fate of the desired lineage. This has been referred as the differentiation window during the hepatic development (Costa et al., 2003; Zaret et al., 2002; Lemaigre et al., 2003). The balance in the numbers of hepatocytes and cholangiocytes from hepatoblasts is achieved by integrated signalling and transcriptional networks. For example, both Notch and activin/TGF-β signaling pathway controls differentiation of hepatoblasts towards a biliary epithelial

phenotype (Kodama et al., 2004; Clotman et al., 2005 Clotman et al., 2006; Navarro-Alvarez et al., 2010), while HGF antagonises biliary differentiation and in conjunction with oncostatin M (OSM) promotes hepatocyte differentiation (Suzuki et al., 2003). In addition, Wnt signaling has been shown to play an important role in hepatic differentiation of fetal liver-derived LPCs or human embryonic stem cells (hESCs) (Hay et al., 2008; Bi et al., 2009).

After ED 16, most of the hepatoblasts are committed to hepatocytic or cholangiocytic lineages. Thus they are no longer bipotential but still proliferating and become unipotent or late progenitor cells. Intrahepatic bile ducts are formed surrounding the large portal vein branches at ED 17. At this time point, the essential lobular arrangement of the liver is complete, but the maturation of the hepatic parenchyma is not complete until several weeks after birth (Van Eyken et al., 1998; Navarro-Alvarez et al., 2010).

Figure 1.1

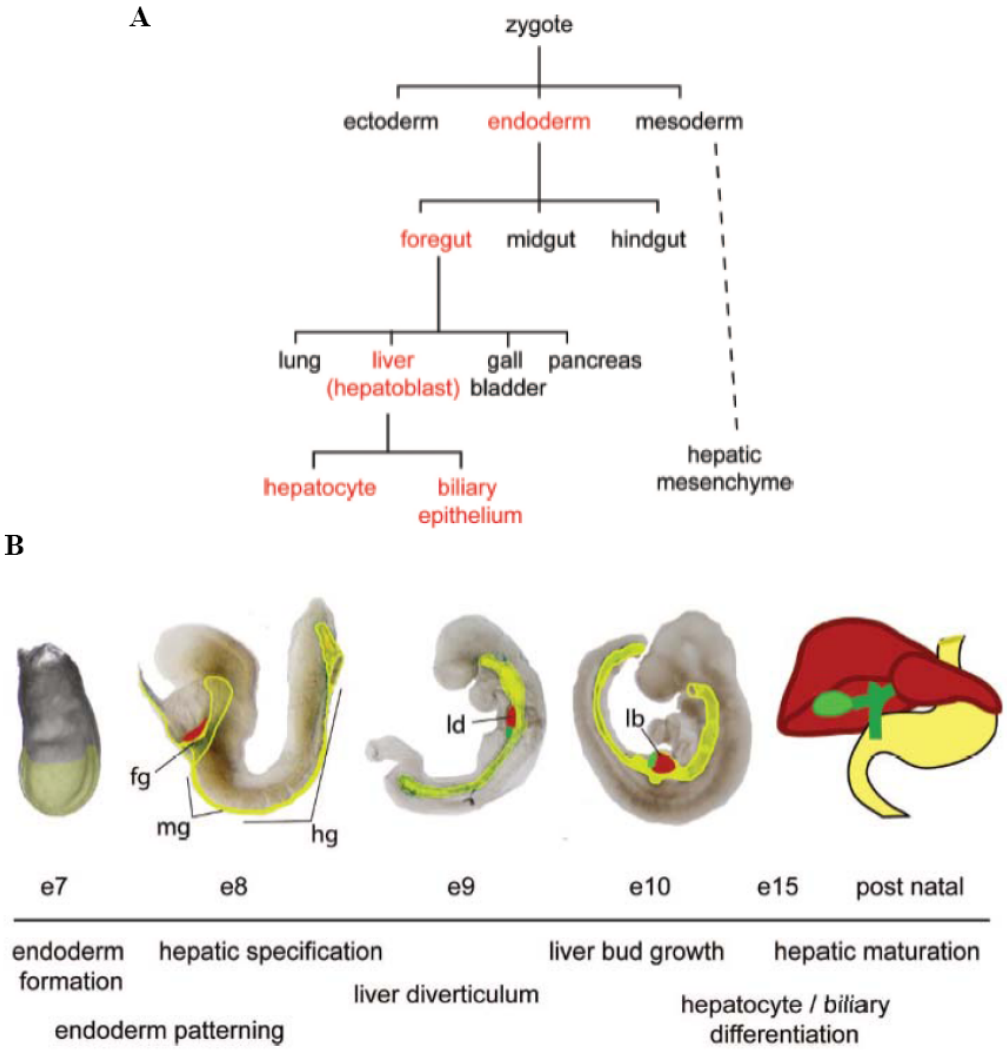


Figure 1.1 Liver development. (A) The cell lineage steps during hepatic development (red) (B) The schematic shows mouse embryos at different stages of development. Around ED 7-ED 8.5, the endoderm is patterned along the A-P axis into foregut (fg) midgut (mg) and hindgut (hg) progenitor domains. Hepatic fate specified in a portion of the ventral foregut endoderm adjacent to the heart at ED 8.5. The liver diverticulum (ld) forms by ED 9 and expands into an obvious liver bud (lb) by ED10. The liver grows, and by ED15 hepatoblasts are differentiating into hepatocyte and biliary cells. Final maturation of the liver is gradual and continues into the postnatal period. Picture copied from Zorn et al., 2008

1.1.2. Liver renewal

The normal adult liver parenchyma consists of mitotically quiescent hepatocytes and cholangiocytes which are the epithelial cells of the bile duct and contribute to bile secretion via net release of bicarbonate and water. Both hepatocytes and cholangiocytes originate from a common endodermal foregut precursor cell. Hepatocytes which are fully differentiated cells generally have a very slow level of turnover. Remarkably, these cells have the ability to re-enter cell cycle in response to mitotic stimuli. Therefore, the adult healthy liver has a unique ability to restore its parenchymal mass and ensure the maintenance of multiple liver functions after different types of injury. Following hepatectomy or acute injury, the regeneration process occurs through the division of mature hepatic cell types, including hepatocytes, biliary epithelial cells, fenestrated endothelial cells, and Kupffer cells and contribute to the regeneration of hepatic tissue (Michalopoulos et al., 2007).

In more severe liver injuries such as acute liver failure, cirrhosis and hepatocellular carcinoma when the function and proliferation of hepatocytes are impaired, liver transplantation is generally the best option to restore liver function. However, the number of donor organs is constantly insufficient to meet demand. Thus, alternative liver treatments are needed. There have been growing interests in LPCs as a potential cell-based therapy for liver diseases.

1.2 Liver stem cells.

1.2.1. Fetal liver stem/progenitor cells (FLSPCs)

As mentioned previously, early fetal liver around ED 12–ED 16 contains hepatoblasts which are bipotent cells derived from endodermal cells. These cells are also known as fetal liver stem/progenitor cells (FLSPCs) due to their similar properties with liver stem/progenitor cells (Shafritz et al., 2006). They express dual markers of the hepatocyte and biliary lineages. In addition, they are capable of differentiating into either hepatocytes or biliary epithelial cells. This bipotent population is regarded as the fetal source of liver progenitor cells and may be useful for liver cell-based therapy. The successful isolation of FLSPCs depends on the liver developmental stages, since cells isolated in late embryological stages will lose the bipotent capacity and are not capable of differentiating into both lineages.

Several markers have been found expressed by FLSPCs may be useful for isolation. Dlk (Tanimizu et al., 2003) and E-cadherin (Nitou et al., 2002) act as useful markers to enrich highly proliferative FLSPCs from fetal liver. Liv2, a hepatoblast marker and Prox1, a transcription factor expressed in early embryonic hepatoblasts, have been shown to be very important during liver development (Watanabe et al., 2002). Both Cd24a and Nope are cell surface markers that were shown to be expressed by murine FLSPCs (Nierhoff et al., 2003). In addition, it has been shown that Sall4 regulates cell fate decision in fetal hepatic stem/progenitor cells not only by inhibiting their differentiation into hepatocytes but also by driving their differentiation toward cholangiocytes (Oikawa et al., 2009).

1.2.2. Liver progenitor cells (LPCs)

LPCs, firstly described as “oval cells” in rodents and “intermediate hepatobiliary cells” in humans, are generally scarce in healthy liver but are stimulated when the regenerative capacity of hepatocyte is impaired. Numerous studies have indicated that when the replacement of hepatic mass loss by hepatocytes is partially or completely precluded, a distinct population of LPCs is induced to proliferate and differentiate towards hepatocytes (Lowes et al. 1999; Akhurst et al. 2001; Knight et al. 2005). Therefore, LPCs have been proposed as a “second line of defense” during liver regeneration. LPCs have also been proposed to arise from the intralobular bile ducts or from the “canals of Hering” which are the smallest, most proximal branches of the biliary tree (Akhurst et al., 2001; Factor et al., 1994; Knight et al., 2005).

Extensive studies have been done to characterize LPCs due to their regenerative role in numerous liver diseases and their great potential for an alternative treatment to liver transplantation. Progenitor cells are also known as transit amplifying cells. They have the ability to proliferate rapidly and to generate more than one differentiated cell type (Potten et al., 1997; Shafritz et al., 2010). The first formal description of LPCs was reported by Farber in 1956. They found a population of nonparenchymal cells appearing in the rat liver after treatment with various carcinogenic agents and described them as “small oval cells with scanty lightly basophilic cytoplasm and pale blue-staining nuclei” (Farber 1956). LPCs have also been shown to represent a heterogeneous population of transit-amplifying progenitor cells ranging from an immature phenotype to mature cholangiocytes and intermediate hepatocytes. In addition, LPCs are bipotential progenitors capable of differentiation into mature hepatocytes and biliary epithelial cells that just like hepatoblasts can differentiate

into both lineages during fetal development (Shafritz et al., 2006).

Many studies have been carried out to assess marker expression on LPCs. (Table1.1) LPCs have been reported to express the immature hepatocyte marker such as α-fetoprotein (αFP); the mature hepatocyte markers such as albumin, cytokeratin (CK 8) and CK18 , and the biliary epithelial cell markers such as γ-glutamyl transferase (GGT), CK7 and CK19 (Radaeva et al., 1995). In addition, LPCs also share some phenotypic characteristics with hematopoietic progenitor cells since they express the hematopoietic stem cell markers such as c-kit tyrosine kinase (c-kit), stem cell antigen-1 (Sca-1), CD34, and CD45 (Petersen et al., 2003, Crosby et al., 2001). Recently, numerous studies suggested other new markers for LPCs such as Delta-like 1 protein (DLK 1) (Yovchev et al., 2007), epithelial cell adhesion molecule (EpCAM) (Okabe et al., 2009) or CD133 (Yovchev et al., 2006). However, universal and specific LPC markers have yet to be established.

The localization of LPCs remains controversial; however, they have been identified in the periductular/ intraportal zone. In addition, they express some hematopoietic stem cell markers, including those found in the bone marrow side population. Therefore, LPCs have been suggested to arise from the bone marrow. Although some studies claimed that a portion of LPCs may arise from the bone marrow and is capable of differentiating into hepatocytes, (Petersen et al., 1999; Oh et al., 2007) many other studies indicated that mouse LPCs are not originated from the bone marrow but from the liver itself (Wang et al., 2003; Menthena et al., 2004). Moreover, a recent study has further confirmed that LPCs are not bone marrow derived in the CDE-fed mice (Lorenzini et al., 2010).

Table 1.1 Various markers expressed by adult liverprogenitor cells (Adapted from Bird et al., 2008)

Adult biliary marker	
Cytokeratin 19 (CK19)	Bisgaard et al. 1993
CK7	Paku et al. 2005
CK14	Bisgaard et al. 1993
γ -Glutamyl transpeptidase (γ GT)	Cameron et al. 1978
Glutathione-S-transferase P (GST-P)	Tee et al. 1992
Muscle pyruvate kinase (MPK)	Akhurst et al. 2005
OV-6 (recognises CK14 and CK19) OV1	Bisgaard et al. 1993
A6	Sanchez et al. 2004
OC.2 and OC.3	Engelhardt et al. 1993
Connexin 43	Hixson and Allison 1985
CX3Cl1	Zhang and Thorgeirsson 1994
CD24	Yovchev et al. 2007
MUC1	Yovchev et al. 2007
Deleted in malignant brain tumour	Yovchev et al. 2007
1 (DMBT1)	Bisgaard et al. 2002
Adult hepatocyte markers	
Albumin	Tian et al. 1997
CK8	Libbrecht et al. 2000
CK18	Libbrecht et al. 2000
α 1-Antitrypsin	Gauldie et al. 1980
Hepatocyte nuclear factor 4 (HNF4)	Nagy et al. 1994
HBD.1	Faris et al. 1991
c-Met	Hu et al. 1993
Fetal hepatocyte markers	
α -Fetoprotein (α FP)	Evarts et al. 1987
Delta-like protein (dlk)	Yovchev et al. 2007
Aldolase A and C	Lamas et al. 1987
c-Met	Hu et al. 1993
Cadherin 22CD24	Yovchev et al. 2007
CD44	Kon et al. 2006
Adult haematopoietic markers	
c-kit	Fujio et al. 1994
CXCR4	Zheng et al. 2006
CD34	Omori et al. 1997
Sca-1	Petersen et al. 2003

1.2.3. Small hepatocyte-like progenitor cells (SHPCs)

Small hepatocyte-like progenitor cells (SHPCs), have also been described in the rat model of LPC induction- partial hepatectomy (PH) in combination with the treatment of pyrrolizidine alkaloid retrorsine (Gordon et al., 2000; Avril et al., 2004). It has been suggested that SHPCs may represent an intermediate or a transitional cell type between LPCs and mature hepatocytes rather than a distinct progenitor cell population (Fausto et al., 2004; Best et al., 2007). SHPCs have characteristics of not only mature adult hepatocytes but also fetal hepatoblasts and LPCs. They resemble differentiated hepatocyte to a certain degree since they express albumin and transferrin; generate bile canaliculi, and store glycogen, which are the functions of differentiated hepatocyte. However, early appearing SHPCs lack (or have reduced expression) of hepatic CYP proteins which is normally expressed on hepatocyte (Gordon et al., 2000). They also express the LPC and fetal liver cell markers: OC2, OC5, and AFP (Gordon et al., 2000). Moreover, they have high proliferative capacity *in vitro* and also following transplantation (Shibata et al., 2006), suggesting that they may have a role in repopulating the liver (Navarro-Alvarez et al., 2010). However, studies from the Forbes group have suggested that they are not an independent progenitor cell population but instead arise from LPCs (Vig et al., 2006).

1.2.4. Animal models of LPC induction

Among these different types of progenitor cells, LPCs have been widely investigated due to their great potential for future cell-based treatments of liver diseases. LPC proliferation can be induced in a number of ways. So far, a variety of liver injury

models to activate LPC proliferation have been established in rodents, including partial hepatectomy (PH) in combination with the treatment of a mitotic inhibitor such as 2-N-acetylaminoflourene (AAF) (Solt et al., 1977). Some toxins such as carbon tetrachloride (CCl4) and allyl alcohol have been used in combination with AAF to induce an LPC response (Petersen et al., 1999). However, the surgery is difficult to perform reproducibly in mice. Recently, choline-deficient diet supplemented with ethionine (CDE) (Akhurst et al., 2001) or the 3, 5-diethoxycarbonlyl-1, 4-dihydrochollinine (DDC) (Preisegger et al., 1999) diet models have become increasingly popular due to their efficacy. In these models, adult hepatocytes fail to respond to growth signals, leading to the activation and rapid proliferation of LPCs which have been described to appear near bile ductules initially and then migrate into the hepatic parenchyma.

CDE diet has been widely used as a model to induce LPC activation. The CDE diet is effective, rapid, and does not require PH to generate substantial numbers of LPCs. The number of LPCs has been shown to increase significantly within 2 weeks (Akhurst et al., 2003). Notably, deregulated LPCs may be a potential source of liver cancer (Lee et al., 2006), with high incidence of hepatocellular carcinoma (HCC) observed in mice fed a choline-deficient diet containing 0.1% ethionine (CDE) for 19 months (Yoshida et al., 1993).

DDC diet is an alternative model to induce LPC activation in the mouse. It has been shown that feeding mice with DDC diet results in persistent proliferation of primitive ductules with poorly defined lumens (Preisegger et al., 1999). However, the precise the mechanism of activating LPCs in this model remains unknown. Similar to other

LPC induction models, DDC-induced atypical ductular proliferation (ADP) originated from the portal tract, spread into the hepatic lobule, and was associated closely with appearance of hepatocytes harboring an antigen (A6), which normally is expressed in biliary epithelium (Preisegger et al., 1999). Moreover, LPCs isolated from the DDC diet-fed mice have shown to repopulate hepatocytes in a transplantation model (Wang et al., 2003).

1.2.5. LPC isolation and LPC line establishment

An in vitro model is essential for further investigating the mechanisms controlling LPC behaviour. Several different techniques for isolating LPCs from rat have been shown recently (He et al., 2004; Pack et al., 1993; Tsao et al., 1984; Yasui et al., 1997; Yaswen et al., 1984). However, few studies have been conducted in mice (Fougere-Deschatrette et al., 2006; Li et al., 2006; Wang et al., 2003). Percoll gradient centrifugation has been reported to successfully separate LPCs, hepatocytes and erythrocytes/ debris (Tirnitz-Parker et al., 2007). It has been commonly used as a method to isolate LPCs from the livers of CDE-fed mice. Generally, the 50% Percoll™ cell layer mainly consists of hepatocytes and the 20% Percoll™ cell layer consists of LPCs along with cells of similar size and density: primarily inflammatory cells and some other minor cell populations such as myofibroblasts and endothelial cells. The 20% Percoll™ layer of cells were collected and the CD45+ hematopoietic cells were depleted by using magnetic beads. Myofibroblasts usually attach to the plates later than LPC, thus they can be removed within 8 to 12 hours after the culturing of isolated LPCs by medium changing. In addition, endothelial cells can be removed by reducing the concentration of growth serum in the culture medium. The

established cultures were not pure but mainly consisted of LPCs (Tirnitz-Parker et al., 2007).

In order to get a purer population of LPCs, several antibodies have been tested to sort cells by flow cytometry and many sub-populations have been described as 'LPCs' in the literature. Numerous LPC surface markers have been identified (Santoni-Rugiu et al., 2005; Yovchev et al., 2007). For example, Ep-CAM, CD133, and DLK1 have been shown to express on the surface of LPCs (Yovchev et al., 2007; Okabe et al., 2009). In addition, CD44 and CD24 which are cell membrane glycoprotein have been reported to express on LPC surface and can be used for isolation of LPCs (Kon et al. 2006; Yovchev et al., 2007). However, the research on identifying specific markers for a pure fraction of LPCs is still a major obstacle in LPC research. The reported markers are also frequently expressed in other cell types. In addition, few reported markers are surface proteins which are suitable for LPCs isolation by cell sorting. In addition, the numbers of different LPC populations are usually extremely low which makes LPC isolation difficult. Moreover, LPCs are heterogeneous and these cells change morphology and express different phenotypic markers when they differentiate (Yovchev et al., 2007).

A variety of reports describing the establishment of biliary epithelial cultures, or the generation of LPC lines from rats have been published (He et al., 2004; Pack et al., 1993; Tsao et al., 1984; Yasui et al., 1997; Yaswen et al., 1984). However, few studies have been conducted in mice (Fougere-Deschatrette et al., 2006; Li et al., 2006; Wang et al., 2003). A recent study has established a LPC line: bipotential murine liver progenitor cells (BMOLs) from the livers of CDE-fed mice

(Tirnitz-Parker et al., 2007). They have demonstrated the establishment of highly enriched LPC cultures and the derivation of an immortalised, non-transformed, clonally derived LPC line from these cultures by using the "plate and wait" method developed for creation of embryonic liver progenitor cell lines (Strick-Marchand et al., 2002). In addition, they have indicated that the BMOLs express both hepatic and biliary markers, and exhibit biopotentiality, i.e. the ability to differentiate into both hepatic and biliary lineages *in vitro*.

1.2.6. LPC differentiation

LPCs play a critical role in the process of liver regeneration since these cells are capable of differentiating along the hepatic lineage into hepatocytes or cholangiocytes (bile duct cells). Several different protocols have been tried to differentiate primary LPCs to investigate the mechanisms governing LPC differentiation. Extracellular growth factors have been shown to be important for LPC differentiation. He et al. (2004) demonstrated that rat LPCs can differentiate into mature hepatocytes in the presence of epidermal growth factor (EGF) and hepatocyte growth factor (HGF) using a two-step induction protocol. Rat LPCs were cultured on a fibroblast feeder layer and supplemented with medium containing EGF for 6 days to induce the LPCs into small hepatocytes. A combination of HGF and EGF were then supplemented for another 6 days to differentiate them further to exhibit morphological, phenotypic and functional characteristics of hepatocytes (He et al., 2004). In addition, the second hepatic compartment: cholangiocytes were also differentiated from rat LPCs in their study. LPCs were grown on fibroblast feeders and HGF alone was supplemented in the medium for 6 days. Biliary epithelium was

classified based on the expression of CK19, and lack of expression of mature hepatocyte markers. In addition, it has been shown that treatment of the rat LPC line with oncostatin M (OSM) induced differentiation into hepatocytes (Okaya et al., 2005). Moreover, the intracellular liver-enriched transcription factors CCAAT/enhancer binding protein α (C/EBPα) and hepatocyte nuclear factor 4α (HNF4α) have also been shown to be important in regulating LPC differentiation into hepatocytes (Dabeva et al., 1995; Nagy et al., 1994; Suetsugu et al., 2008).

Few studies have been done to differentiate mouse LPCs into both lineages. Cultured LPCs in matrigel have been proved to favour biliary lineage differentiation (Li, et al., 2002). In addition, Dimethyl sulfoxide (DMSO) has been identified to maintain hepatocyte differentiation phenotype in vitro (Spagnoli, et al., 1998). Li, et al (2006) have demonstrated that medium supplemented with 2 % matrigel and 2 % DMSO can differentiate mouse LPCs into both lineages. In addition, they also showed that culturing mouse LPCs on matrigel-coated plates with supplements of EGF can lead to murine LPC differentiation toward a hepatic fate, whereas supplementing the medium with HGF can lead to biliary lineage cell differentiation.

It has been shown that LPCs can also differentiate into pancreatic cells or intestinal epithelium (Yang et al., 2002). However, LPCs may also have a pathological role since they have been implicated as precursors of hepatocellular carcinoma and cholangiocarinoma. (Sell et al., 1989)

Notably, further *in vivo* research needs to be conducted to investigate LPC differentiation since other extracellular environmental factors may influence LPC

differentiation. Factors secreted by surrounding cells types, such as inflammatory, Kuppfer and hepatic stellate cells have also been proposed to play an important role in stimulating LPC proliferation and differentiation (Erker et al., 2008). Furthermore, hepatic stellate cells have been shown to induce differentiation of LPCs into mature hepatocytes (Chen et al. 2009).

1.3 Liver progenitor cell (LPC) niche

1.3.1. Location

During embryonic development, hepatoblasts give rise to primitive intrahepatic bile ducts which connect parenchymal hepatocytes with the biliary system. These primitive intrahepatic bile ducts correspond to the canals of Hering and terminal bile ductules of adult livers may constitute the niche for LPCs (Van Eyken et al., 1998; Navarro-Alvarez et al., 2010).

It is proposed that a restricted locale which is called “liver progenitor cell niche” surrounds LPCs and contributes to the support and regulation of LPC behaviour (Fuchs et al., 2004; Spradling et al., 2001). Within this niche, the direct contact between LPCs and supporting cells are thought to be responsible for maintaining the balance between progenitor cell expansion and differentiation (Nusse et al., 2008). This microenvironment comprises the extracellular matrix, epithelial and non-epithelial resident liver cells, recruited inflammatory cells, and a variety of growth-modulating molecules (Santoni-Rugiu et al., 2005). However, the precise location of the progenitor cell niche and the cellular components of this niche have not been well defined yet.

1.3.2 Supporting cells in the LPC niche

So far, several potential candidates in the LPC niche have been demonstrated, including macrophages, myofibroblast, endothelial cells, and hepatic stellate cells. It has been shown that LPCs are closely accompanied by a cellular niche composed of

macrophages, myofibroblast, and endothelial cells throughout the progenitor cell response in rodent models of LPC induction and human liver disease (Lorenzini et al., 2010).

In addition, hepatic stellate cells, portal fibroblasts, and myofibroblasts have been proposed to play an important role in constituting the LPC niche. Hepatic stellate cells have been demonstrated to have a close physical relationship with LPCs and also function as supporting cells in the niche, playing an important role in LPC expansion and differentiation (Roskams et al., 2008; Zhang et al., 2009; Braun et al., 2003; Paku et al., 2001). Moreover, co-culture of hepatic stellate cells and LPCs cells results in increase in LPC proliferation and LPC differentiation into hepatocytes (Nagai et al., 2002; Lin et al., 2008; Deng et al., 2008). However, whether cell-cell contact is required for this mechanism remains unclear. Even so, soluble mediators produced by activated hepatic stellate cells have been suggested to mediate LPC proliferation (Santoni-Rugiu et al., 2005). Interestingly, quiescent hepatic stellate cells may have a counter effect that retains progenitor cells in the niche (Clouston et al., 2009). The stellate cells, which respond to liver injury through activation, may have different effects upon LPCs depending on liver activation status.

The role of LPC neighbouring inflammatory cells in supporting LPC behaviour has also been proposed recently (Viebahn et al., 2008; Libbrecht et al., 2000; Viebahn et al., 2008). Recent studies also demonstrated that macrophages increased in number and surrounded around LPCs when LPC expansion was induced. Moreover, it has been shown that most of these macrophages are bone marrow derived (Lorenzini et al., 2010). Furthermore, cytokines produced by inflammatory cells such as the

tumour necrosis factor α (TNFα), interferon gamma (IFNγ), TNF-like weak inducer of apoptosis (TWEAK), lymphotoxin beta (LTβ), interleukin-6 (IL-6), and transforming growth factor beta (TGF-β) have been shown to be crucial for the LPC response (Knight et al., 2005; Viebahn et al., 2010). Viebahn et al. (2010) have shown that both infiltrating (CD11b+) and resident (CRIg+) macrophages are present around LPC response and these are the most important cytokine producers since they contribute to TNFα, IL-6, TGFβ and TWEAK. They proposed that liver macrophages in combination with these cytokines are important for the induction of LPC expansion. Moreover, they also proposed that during LPC response, LPCs themselves recruit macrophages to the damaged liver as LPCs express CCL2 and CX3CL1. Increased hepatic expression of chemokines such as CCL2 and CX3CL1 were observed in LPC induction models, suggesting the role in mediating LPC response. It has been proved that CCL2 and CX3CL1 are expressed predominantly by infiltrating macrophages and crucial for attracting macrophages to the injured liver (Karlmark et al., 2008). Furthermore, they demonstrated that the LPC line: BMOLs can attract infiltrating (CD11b+) macrophages isolated from the livers of CDE-fed mice. More research is required to further analyze the role of macrophage in supporting LPC proliferation and differentiation.

1.3.3. The role of extracellular matrix in the LPC niche

It has been proposed that cell-matrix and cell-cell interactions are important in regulating progenitor cell proliferation and differentiation within the LPC niche (Spradling et al. 2001). The basement membrane of bile ducts is composed of laminin and type IV collagen (Terada et al., 1994; Yasoshima et al., 2000). It is proposed that signals delivered by extracellular matrix (ECM) proteins, such as laminin and type IV collagen may also take part in regulating and supporting LPC behaviour. Previous studies have demonstrated that a laminin rich basement membrane is strongly associated with the LPC response in 2-acetylaminofluorene model of liver injury in rats (Paku et al., 2004). In addition, it has been found that laminin, but not type I and IV collagen was heavily deposited in sheath which closely surrounds LPCs in rodent models of LPC induction and human liver disease, while only appears prominently around portal vessel with weak staining in the control liver (Lorenzini et al., 2010). Interestingly, LPCs only extend as far as laminin sheath, suggesting the important role of laminin in constituting the LPC niche (Fig 1.2).

Other research also indicated that LPCs appear partially differentiated when they are beyond the limit of the deposited laminin sheath (Van Hul et al., 2009), suggesting that a specialized matrix may be required for the expansion but not differentiation of LPCs. Laminin has been shown to facilitate LPCs to maintain an undifferentiated phenotype (Lorenzini et al., 2010). However, further research is required to investigate the role of laminin in regulating LPC proliferation and differentiation.

1.3.4. The role of integrins in the LPC niche

Integrins play an important role in mediating cell adhesion to a basal lamina. They have also been proposed as additional important factors in regulating LPC proliferation and differentiation within the LPC niche. Both neural stem cells and epidermal stem cells have been found to express high levels of integrin β1 (Campos et al., 2004; Jensen et al., 1999; Zhu et al., 1999). The niche may retain the progenitor cells by providing extracellular matrix (ECM) ligands for the integrin receptors on the surface of progenitor cells (Watt et al., 2000; Navarro-Alvarez et al., 2010). Integrin α6β1 has been identified as a laminin receptor (Delwel. et al. 1996). Theoretically, the interaction between laminin and integrin α6β1 should be required to maintain LPCs in an undifferentiated state within the LPC niche. It has been shown that integrins (α2, α3, α5, α6, and α9, which dimerize with β1) and basement membrane components such as laminin and type IV collagen are predominantly expressed in human biliary epithelium (Yasoshima et al., 2000). Moreover, previous studies also examined the expression of integrin CD49f (integrin 6α) in non-parenchymal cell fraction isolated from normal human, acutely injured and chronically injured liver. They have found that the proportion of cells expressing the integrin 6α was higher in diseased liver than in normal liver, suggesting a possible but unproven role of integrin 6α in LPC expansion (Laurson et al., 2007). However, little research has been carried out to investigate the expression of integrins in rodent models of LPC induction. Unpublished studies from the Forbes group (Clayton) have demonstrated different expression level of integrin subunits during the differentiation of LPCs into hepatocytes. The expression of integrin β1 increases when LPCs differentiate into biliary progenitor cells and when they differentiate into to mature

hepatocytes, they begin to express integrin α1β3. These findings suggested that integrins may be essential for cell differentiation decision of the LPCs. Clearly, further research has to be done to analyze the role of integrins in regulating LPC proliferation and differentiation.

Figure 1.2

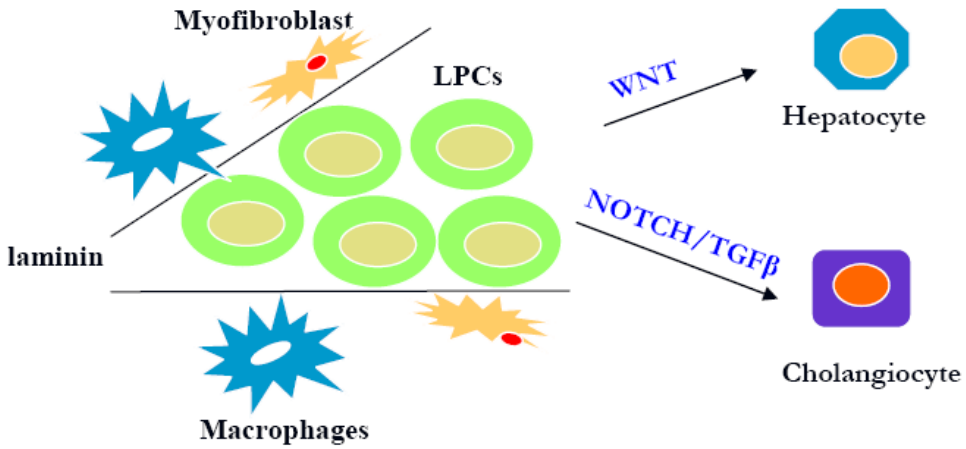


Figure 1.2 Heptic progenitor cell niche It has been proposed that laminin and other supporting cells such as macrophages and myofribroblast might be essential component of the LPC niche to regulate LPC proliferation and differentiation. Laminin might be required to maintain LPCs in an undifferentiated state within the LPC niche. Once LPCs leave the laminin niche, they become differentiated.

1.4. Galectins

1.4.1 General Backgrounds

Galectins, widely distributed in mammals, birds, amphibians, fish, nematodes, sponges, and some fungi, contain a carbohydrate-recognition domains (CRD) which is a beta-sheet and consists of about 130 amino acids. Galectins are a family of proteins defined by having at least one characteristic carbohydrate recognition domain (CRD) with an affinity for beta-galactosides through a conserved sequence in the carbohydrate-binding site (Barondes et al., 1994). The CRD of galectins is responsible for carbohydrate binding and is able to recognize various carbohydrates attached to proteins and lipids on cell surfaces and extracellular matrices, known as glycoconjugates. Galectins have many intra- and extracellular functions through glycoconjugate-mediated recognition, varying from the regulation of cell adhesion and promotion of cell–cell interactions.

1.4.2 The galectin family

Fifteen mammalian galectins have been identified and classified into three groups according to their biochemical structure (Hirabayashi et al., 1993). The Prototype galectins (galectin-1.-2.-5.-7.-10.-11.-13.-14 and -15) consist of one CRD and a short N-terminal sequence; they normally exist as monomers or non-covalent homodimers (Fig 1.2 A). The Tandem repeat type of galectins (galectin-4,-6,-8,-9, and-12) contain two non-identical CRDs linked via a short peptide sequence up to 70 amino acids (Fig 1.2 B). The Chimera type of galectin (galectin-3) consists of one CRD and one non-lectin domain contains unusual tandem repeats of short amino-acid stretches fused onto the CRD, and a short NH_2-terminal domain (Figure 1.2 C).

Figure 1. 3

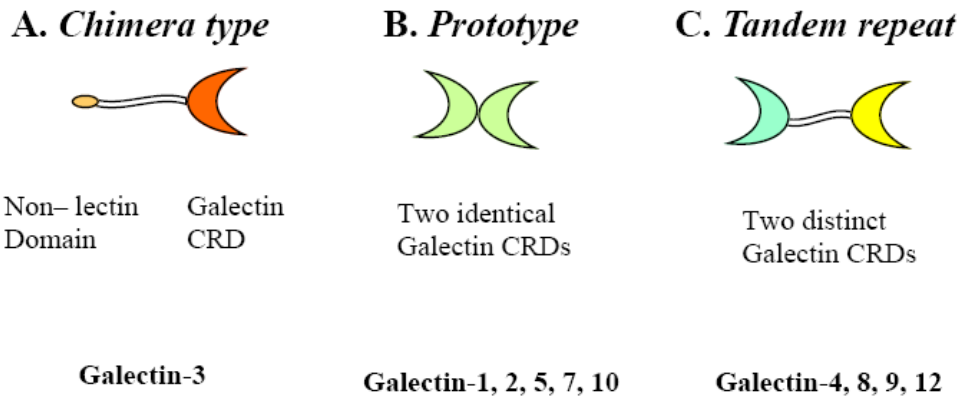


Figure 1.3 Galectin Family (A) The Chimera type galectins cross-link one CRD and a non lectin– domain. (B) Some prototype galectins present as dimers, crosslinking two homologous CRD. (C) The Tandem repeat type of galectins cross-link two non-identical CRD.

Generally, galectins are either bivalent or multivalent according to their carbohydrate-binding activities. Some Prototype galectins (One-CRD) are found as dimers. Tandem repeat types of galectins (Two-CRDs) consist of two carbohydrate binding sites, and galectin-3 normally forms oligomers when it binds to multivalent carbohydrates. The CRD of galectins can recognize adjacent oligosaccharides and different galectins can bind to different sets of oligosaccharides. Galectins have been found to crosslink cell-surface glycoconjugates and trigger a cascade of transmembrane signalling events which are important for apoptosis and cell-cycle progression. In addition, galectins can cause the clustering of multiple multivalent glycoconjugates which results in a lattice formation.

Although there is no evidence of a signal sequence in galectins, which is required for protein secretion through the classical secretory pathway, some galectins have been found in the extracellular space and secreted by cells, probably through a non-classical secretory pathway (Cooper et al., 1990; Hughes et al., 1999). Galectin-3 can also relocalize to the plasma membrane and then become a part of vesicles which extrude from the plasma membrane, but the signals which control this are still unknown (Liu et al., 2002).

Galectins have been found widely distributed in various tissues or specific to certain types of tissue. The expression of galectins have been found to be regulated during the development and altered under different physiological or pathological conditions (Chiariotti et al., 2002).

1.5 Galectin-3

1.5.1 Structure

Galectin-3 , a 30 kDa unique chimeric gene product, consists of three structural domains: (A) a NH_2-terminal domain of 12 amino acid that contains a serine phosphorylation site; (B) a repeated collagen-like sequence rich in glycine, proline, and tyrosine; and (C) a COOH-terminal carbohydrate recognition domain (CRD) consisting 140 amino acid residues (Figure 1. 4).

As all galectins, galectin-3 is composed of a carbohydrate-recognition-binding domain (CRD) about 130 amino acids that enable specific binding of β-galactosides (Liu. et al., 1990; Hughes et al., 1994; Barondes et al., 1994). The C-terminal domain of galectin-3, within the carbohydrate-recognition domain includes NWGR; this motif is highly conserved within the BH1 domain of the Bcl-2 family proteins, and it was shown to be responsible for the anti-apoptotic activity of both Bcl-2 and galectin-3 (Yang et al., 1996). (Fig. 1.4)

The N-terminal domain (ND) of galectin-3 is composed of 110 – 130 amino acids. This domain is involved in the oligomerization of galectin-3. The oligomerization results in the formation of a galectin- 3 molecule that possesses multivalent CRDs and enables galectin -3 to mediate crosslinking of its ligands (Barondes et al., 1994, Kasai et al., 1996, Hughes et al., 1997). The ND has been also implicated in secretion of galectin-3 outside of cells (Menon et al., 1999). Deletion of these first 11 amino acids (following the first methionine) blocks secretion of galectin-3 (Gong et al., 1999). In addition, the phosphorylation at the position Ser6 of galectin-3 strongly

affects its sugar binding affinity (Mazurek et al., 2000). Moreover, the presence of both phosphorylated and unphosphorylated form of galectin-3 has been reported for the first time in murine 3T3 fibroblasts; phosphorylated galectin-3 was found in both the cytoplasm and the nucleus, whereas unphosphorylated form was found exclusively in the cytoplasm (Cowles et al., 1990). These findings suggested requirement of phosphorylation for transport of galectin-3 into the nucleus.

Figure 1. 4

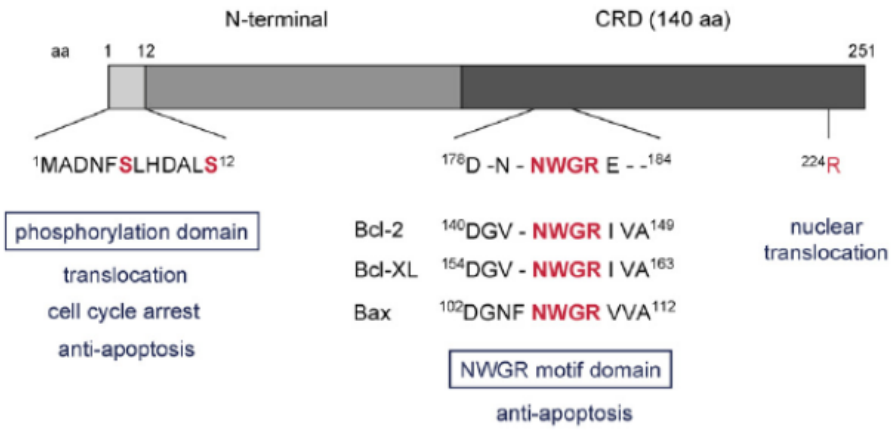


Figure 1.4 Structure and functional domain of galectin-3 picture copied from Fukumori et al., 2007

1.5.2 Distribution and basic function

Galectin-3 has been found in various types of cells, including epithelial cells, fibroblasts, dendritic cells, and inflammatory cells (Flotte et al., 1983). In particular, galectin-3 is highly expressed on activated macrophages and monocytes (Liu et al., 1995). The expression of galectin-3 has been found to correlate with cancer aggressiveness and metastasis (Takenaka et al., 2004; Iurisci et al., 2000; Buttery et al., 2004; Kim et al., 1999). For example, galectin-3 has been found expressed in thyroid, breast, and colon carcinoma (Xu et al., 1995; Castronovo et al., 1996; Schoeppner et al., 1995). The over-expression of galectin-3 has been reported in hepatocellular carcinoma. In addition, galectin-3 is involved in the tumour progression and related to the biomarker for tumor development and could be the prognosis of hepatocellular carcinoma which suggests that galectin-3 can be used as a potential therapeutic target (Matsuda et al., 2008).

Galectin-3 has been reported to be involved in various biological phenomena, including cell growth and proliferation (Moutsatsos *et al.,* 1987; Inohara *et al.,* 1998), adhesion (Kuwabara and Liu, 1996; Inohara and Raz, 1995; Inohara *et al.,* 1996), cell survival (Yang *et al.,* 1996; Akahani *et al.,* 1997), and cell cycle control (Kim et al., 1999; Lin et al., 2000).

Galectin-3 can be found on the cell surface, extracellularly and intracellularly, and in both the cytoplasm and nucleus. The nuclear localization of galectin-3 is well documented; however, the well-characterized nuclear localization signal and the mechanism by which the protein is sequestered in nuclei remain unknown. It has

been suggested that galectin-3 shuttles between the cytoplasm and nucleus on the basis of targeting signals that are recognized by importin(s) for nuclear localization and exportin-1 (CRM1) for nuclear export.

Gong et al. reported that the first eleven residues of galectin-3 governed its nuclear import (Gong et al., 1999); however, later study rather implied the involvement of the CRD in nuclear import and retention of galectin-3 inside the nuclei (Spector et al., 2001). Moreover, Nakahara et al. identified a sequence in residues 223-228 (HRVKKL) of galectin-3, within CRD, is similar to the lysine/arginine-rich nuclear localization signal (NLS) which is found in many nuclear proteins. Furthermore, galectin-3 has been shown to bind to importin-α directly (et al., 2006).

Galectin-3 export is rapid and selective process that proceeds via leptomycin (LMB) - inhibitable pathway (Tsay et al., 1999). The addition of LMB which is a drug shown to bind to and inhibits the interaction of exportin-1 (CRM1) with leucine-rich NESs contributes to the retention of galectin-3 in the nucleus. The NES (residues 241-256) is found in the hydrophobic β-sandwich motif of the CRD and is highly conserved in the galectin-3 homologues of various species. On the other hand, it was found that only phosphorylated galectin-3 is transported out of nuclei (Tsay et al., 1999). Mutant at the position ser6 of ND which is the major site of phosphorylation inhibit galectin-3 exported from the nucleus to cytoplasm, suggesting that phosphorylation of galectin-3 is required for exporting galectin-3 from the nucleus to the cytoplasm.

1.5.3. Intracellular functions of galectin-3

Intracellular galectin-3 has been found shuttling between nucleus and cytoplasm (Davidson et al., 2002) and engaged in various basic cellular functions such as pre-mRNA splicing, cell growth, cell apoptosis, and cell-cycle regulation (Dagher et al., 1995; Vyakamam et al., 1997; Wang et al., 2004). Several proteins have been shown to interact with galectin-3 such as Bcl-2 (Yang et al., 1996; Akahani et al., 1997), Gemin4 (Park et al., 2001), Ras (Elad-Sfadia et al., 2004), Akt/PKB (Oka et al., 20055), and β–catenin (Shimura et al., 2004) have been identified.

Galectin-3 has been identified to have a similar sequence to B cell lymphoma 2 (BCL2), which is a suppressor of apoptosis (Yang et al., 1996). Galectin-3 and BCL2 both contain an Asp-Trp-Gly-Arg (NWGR) motif in the carboxyl- terminal part of the molecule which is a sequence essential for the regulation of apoptosis. Generally, substitution of glycine to alanine in this motif stimulates the anti-apoptotic activity of galectin-3 (Akahani et al., 1997). However, the motif in galectin-3 functions has not been completely investigated. Moreover, the direct interactions between these two proteins and their intracellular associations have also not been established. However, it has been proposed that galectin-3 has a regulatory role in anti- apoptosis by binding to BCL2 or mediating the transport of BCL2 to mitochondria (Liu et al., 2002).

Galectin-3 has been shown to interact with Gemin4 which is a component of a macromolecular complex involved in pre-mRNA splicing. This suggests the role of galectin-3 in the splicing pathway (Park et al., 2001).

Galectin-3 has been found interacting with oncogenic Ras, especially KRAS (Paz et al., 2001; Elad-Sfadia et al., 2004). These interactions promote Ras-mediated signal transduction such as RAF1, extracellular signal-regulated kinase (ERK) 1/2, phosphatidylinositol-3-kinase (PI3K) and the serine/threonine kinase Akt, which play essential roles in cell proliferation, differentiation, survival, and death (Elad-Sfadia et al., 2004).

Galectin-3 has been shown to be involved in Wnt/β-catenin signalling. Galectin-3 can bind to β-catenin, which is the effector component of Wnt signalling pathway. Phosphorylation of casein kinase I (CKI) and glycogen synthase kinase 3β (GSK3β) result in activation of β-catenin. Galectin-3 has been found to co-localize β-catenin in the cell nucleus and stimulate cyclin D1 or c-myc expression which are important for cell cycle regulation and promoting cell cycle progression (Shimura et al., 2004; Shimura et al., 2005).

1.5.4. Extracellular functions of galectin-3

Galectin-3 has also been found to be present on the cell surface and in the extracellular compartment. The biological activities of extracellular galectin-3 mainly involve its interactions with various β-galactoside containing glycans via CRD. Galectin-3 can bind to cell-surface glycoconjugates that contain galactose-containing oligosaccharides. Inhibitors of galectin-3 that function in the extracellular compartment have been identified, eg. lactose (Platt et al., 1992; Inohara et al., 1994; Pienta et al., 1995). Various glycoproteins such as laminin (Ochieng et al., 1995; Wang et al., 1992; Kuwabara et al., 1996), collagen IV

(Ochieng et al., 1998), fibronectin (Matarrese et al., 2000; Sato et al., 1992), hensin (Hikita et al., 2000), and elastin (Ochieng et al., 1999) have been shown as extracellular receptors for galectin-3. In addition, integrin α1β1 (Ochieng et al., 1998; Andre et al., 1999) and CD98, a transmembrane protein which will be introduced later, have also been proved as important ligands for galectin-3 (Dong et al., 1996).

Galectin-3 has been found to play a key role in modulating cell to cell, and cell to extracellular matrix interaction. Furthermore, it is now proposed as a mediator of signal transduction events on the cell surface which is summarized in Figure 1.5. Galectin-3, as other receptor-ligand systems, can cross link surface glygoproteins and transduce signals to regulate gene expression (Ochieng et al., 2004). Galectin-3 is also involved in regulating a variety of extracellular processes such as kidney development, angiogenesis, neuronal functions, tumor metastasis, autoimmune disorders, endocytosis and possibly exocytosis (Bao et al., 1995; Liu et al., 1993; Raz et al., 1987; Nagnia et al., 2000; Lee et al., 1998).

Figure 1. 5

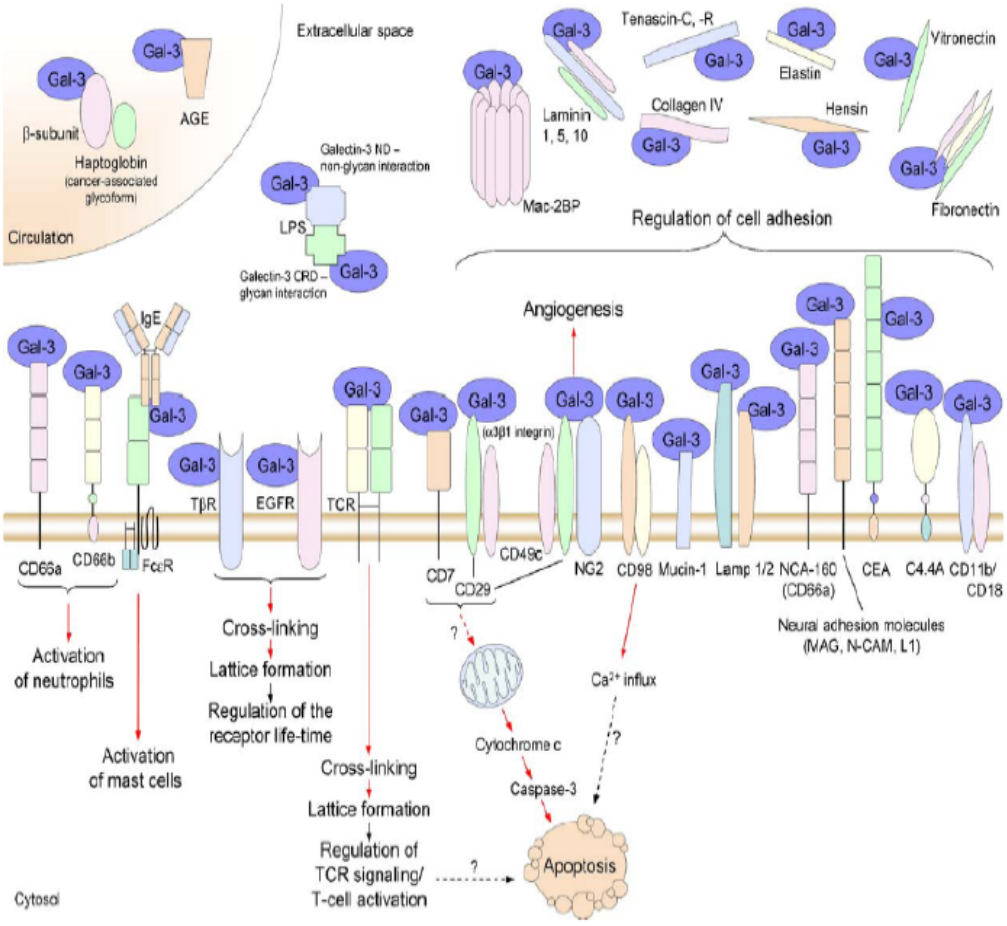


Figure. 1.5. The extracellular functions of galectin-3. Red arrows indicate positive effects. AGE: advanced glycation end products, C4.4A: the GPI-anchored glycoprotein C4.4A, CEA: carcinoembryonic antigen, EGFR: epidermal growth factor receptor, L1: neural adhesion molecule L1, Lamp 1/2: lysosome associated membrane protein 1/2, LPS: lipopolysaccharide, Mac-2BP; Mac-2 binding protein, MAG: myelin associated glycoprotein, N-CAM: neural cell adhesion molecule, NCA-160: non-specific crossreacting antigen 160, NG2: the transmembrane chondroitin sulfate proteoglycan NG2, TβR: transforming growth factor β receptor, TCR: T cell receptor. Picutre copied from Dumic et al., 2006

1.5.5. The regulating role of galectin-3 in cell adhesion

Galectin-3 regulates cell-cell and cell-matrix adhesive interactions, which are crucial for normal cellular motility and polarity and during tissue formation. It has been shown that epithelial cells, which lack galectin-3 expression, interact poorly with their extracellular matrices. In addition, it has been demonstrated that galectin-3 is related to several disease states such as tumour progression. For example, the migration of human metastatic breast tumour cell-lines through a Matrigel barrier in a Transwell assay was increased by a moderate sub-micromolar concentration of galectin-3 (Le et al., 1996). Moreover, galectin-3 may regulate cell growth by mediating cell adhesion. For example, it has been shown that galectin-3 promotes neural cell adhesion on laminin and neurite growth (Penka et al., 1998).

Generally, galectin-3 can modulate cell adhesion on extracellular matrix either positively or negatively. For example, breast cancer cells with high expression of galectin-3 interact well and spread very rapidly on ECM proteins compared to those with low or no galectin-3 expression (Warfield et al., 1997; Matarrese et al., 2000; Honjo et al., 2001). Galectin-3 significantly increased the adhesion of human neutrophils to various substrata (Ohannesian et al., 1994; Kuwabara et al., 1996). However, galectin-3 has been shown to reduce the adhesion of myoblasts and kidney epithelial cells to laminin (Sato et al., 1992).

A model of cell adhesion regulated by galectin-3 has been proposed by Hughes (Hughes et al., 2001). Galectin-3 may modulate cell adhesion through either direct or indirect effects which involves integrin activation. (Figure 1.6) The regulation of cell

adhesion by galectin-3 may be positive or negative. Monovalent galectin-3 may ligate with glycoproteins of either an integrin or extracellular matrix protein, thus weakening the adhesive interaction by steric hindrance. (Fig. 1.6 A) On the other hand, a functionally bivalent galectin-3 can also act synergistically on cell-cell adhesions and cell-matrix adhesions to stimulate adhesion by cross-linking cell-surface and matrix molecules (Fig. 1.6 B) (Hughes et al., 2001). This mechanism may also involve integrin activation. Galectin-3 may regulate integrin activation which is essential for cell adhesion to ECM by binding to the extracellular domains of integrin subunits.

Figure 1. 6

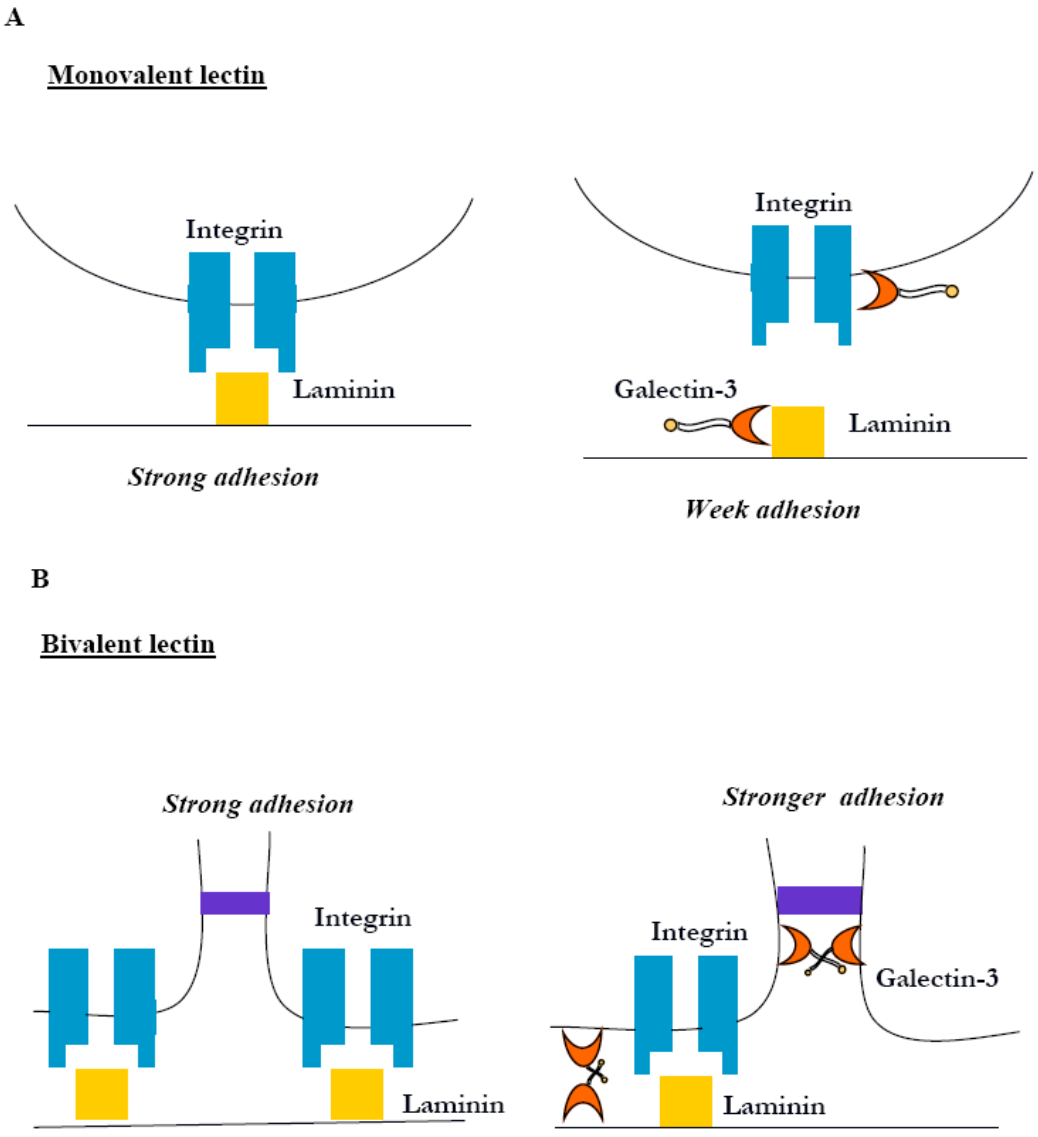


Figure 1.6 Galectin-3 might modulate cell adhesion by regulate adhesive potential between cell-cell or cell-matrix positively or negatively. (A) Monovalent Galectin-3 binds to either integrin or laminin, thus weakening the adhesive interaction by steric hindrance. (B)Bivalent Galectin-3 can act synergistically in cell-cell or cellmatrix adhesion, thus stimulate adhesion. Picture adapted from the review by Hughes, R.C. (2001)

1.5.6. Galectin-3 plays a regulatory role in cell growth and cell cycle regulation

The role of galectin-3 in regulating cell growth has been demonstrated by numerous studies. For example, transfecting the human T lymphoma Jurkat cells with galectin-3 can lead to faster cell growth than control transfectants not expressing galectin-3, especially under suboptimal growth condition (Yang et al., 1996). Furthermore, transfecting the human breast cancer cells with antisense galectin-3 cDNA significantly decreased cell proliferation (Van den Brule et al., 1997). Moreover, it has also been indicated that galectin-3 transfectants of Jurkat cells mentioned above can survive significantly longer than control transfectants when they were treated with anti-Fas receptor antibody which can cross-links Fas receptor and causes apoptosis (Yang et al., 1996). Also, overexpression of galectin-3 in human breast carcinoma cells inhibited apoptosis (Akakani et al., 1997). These findings suggested the function of galectin-3 in either promoting cell growth or inhibiting cell death.

Nonetheless, the mechanism of galectin-3 regulation of cell growth has not been completely investigated. As mentioned above, intracellular galectin-3 has been found to bind components such as Bcl-2 and Ras to regulate cell apoptosis and cell proliferation. For example, it has been shown that galectin-3 can stimulate proliferation of rat hepatic stellate cells through the MEK1/2 - ERK1/2 signaling pathways (Maeda et al., 2003). On the other hand, it is also possible that extracellular mechanisms are involved. Galectin-3 may regulate cell growth by binding cell surface glycoconjugates to modulate cell adhesion on extracellular matrix. It has also been shown that adding galectin-3 exogenously can stimulate

growth of fibroblasts (Inohara et al., 1998), mesangial cells (Sasaki et al., 1999), and promote outgrowth of neurites from dorsal root ganglia explants (Pesheva et al., 1998).

Cell proliferation is known to be controlled by cell cycle regulators. For example, Cyclin D1 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for G1 to S cell cycle transition. It has been shown that cyclin D1 interacts with retinoblasma tumour suppressor protein (Rb) and the expression of this gene is regulated positively by Rb. P21 (WAF1), a potent cyclin-dependent kinase inhibitor (CKI) which binds and inhibits the activity of cyclin E/CDK2 and cyclin D/CDK4 complexes directly functions as a regulator of cell cycle progression from G1 to S phase. The expression of p21 is controlled by the tumour suppressor protein p53 and cell growth arrest by p21 can promote cellular differentiation. Moreover, p16, Cyclin-dependent kinase inhibitor 2A, is a tumour suppressor protein which plays an important role in regulating cell cycle. P16 functions as a cell cycle G1 control through the regulatory roles of CDK4 and p53 in cell cycle G1 progression. Mutations in p16 increase the risk of developing multiple cancers, thus p16 is known to be an important tumour suppressor gene. It has been demonstrated that increased p16 gene expression reduces the proliferation of stem cells (Krishnamurthy et al., 2006). (Figure 1.7)

The role of galectin-3 in regulating the expression of cell cycle regulators has also been demonstrated. Galectin-3 has been shown to activate the cyclin D1 promoter in human breast epithelial cells (Lin et al., 2002). In addition, galectin-3 knockdown in human prostate cancer cells led to cell-cycle arrest at G1 phase, up-regulation of p21,

Figure 1.7

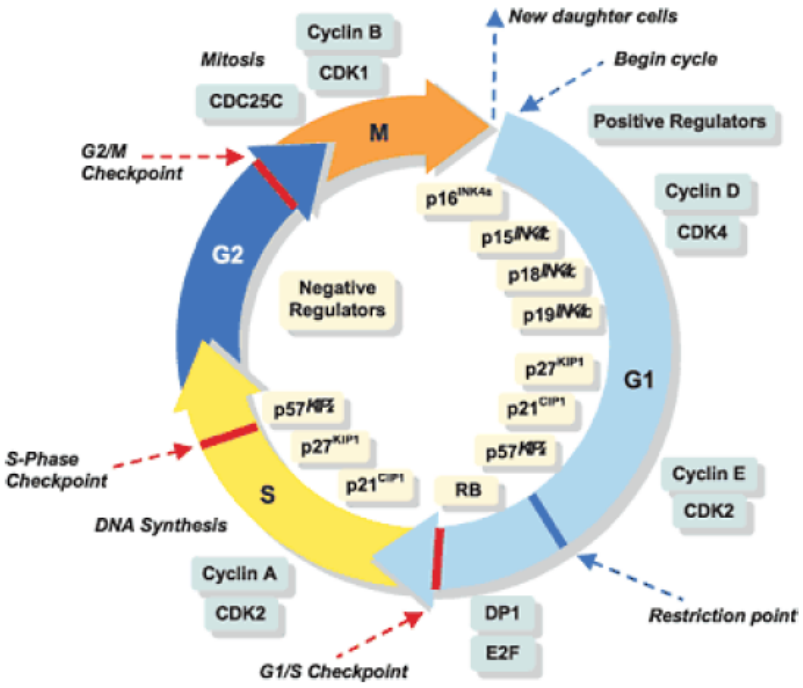


Figure 1.7 Cell cycle regulated by cyclins and cell cycle inhibitors. copied from Kong et al., 2003

and hypophosphorylation of the retinoblasma tumor supressor protein (pRb) (Wang. et al., 2009). Moreover, galectin-3 binds to β-catenin which is an intracellular protein implicated in cell cycle regulation and colocalizes with β-catenin in the nucleus to stimulate gene expression of cyclin D1 and c-myc which are important for cell cycle progression (Shimura et al., 2004).

The mechanism by which galectin-3 regulates cyclin D1 remains unclear. It is believed that the activation of the cyclin D1 promotor can be triggered by growth factors targeting several signal transduction pathways such as the Ras-Raf-p42/p44MAPK (Albanese et al., 1995; Lavoie et al., 1996; Lee et al., 1999). The growth factor activation of cyclin D1 promoter is mediated by multiple binding sites on the cyclin D1 promoter (Guttridge et al., 1999; Hinz et al., 1999; Joyce et al., 1999; Matsumura et al., 1999; Nagata et al., 2001; Watanabe et al., 1996). On the other hand, cell adhesion signalling has been proved to be required for cyclin D1 transcription (Le Gall et al., 1998; Zhu et al., 1996). It has been shown that cell adhesion-mediated cyclin D1 promoter activation appears to occur through focal adhesion kinase (FAK) (Zhao et al., 1998) and integrin-linked kinase (ILK) pathways (D'Amico et al., 2000). However, it has been demonstrated that galectin-3 induces cyclin D1 promoter activity in human breast epithelial cells independent of cell adhesion. They proposed that galectin-3 may be involved in the enhancement/stabilization of nuclear protein-DNA complex formation at the CRE site of the cyclin D1 promoter (Lin et al., 2002)

1.5.7. The role on galectin-3 in liver biology

Little research on the function of galectin-3 in the physiologic and pathologic processes of liver has been published (Henderson et al., 2006; Shimonishi et al., 2001; Kristensen et al., 2000; Hsu et al., 1999; Santucci et al., 2000). Galectin-3 expression has been found to increase significantly in the regenerative nodules of cirrhotic liver tissues and during hepatocellular carcinoma (Hsu et al., 1999). In addition, inhibition of galectin-3 on the surface of cancer cells results in reduced cell attachment to laminin and metastasis in the liver (Inufusa et al., 2001).

The role of galectin-3 in modulating the growth of hepatic stellate cells has been proposed. Galectin-3 has been found to modulate the growth of fibroblasts by interacting with their surface glycoconjugates (Inohara et al., 1998). Furthermore, it has been shown that galectin-3 can stimulate proliferation of rat hepatic stellate cells through the MEK1/2 - ERK1/2 signaling pathway (Maeda et al., 2003).

The role of galectin-3 in liver fibrosis has also been demonstrated. It has been shown that the expression of galectin-3 was significantly up-regulated in stellate cells when they transdifferentiate into myofibroblasts, a process called "self-activation" (Maeda et al., 2003). In addition, previous studies showed that disruption of the galectin-3 gene blocks myofibroblast activation and significantly attenuated liver fibrosis (Henderson et al., 2006). Furthermore, in vivo siRNA knockdown of galectin-3 inhibited myofibroblast activation after hepatic injury may therefore provide an alternative therapeutic approach to the prevention and treatment of liver fibrosis (Henderson et al., 2006).

1.6. Integrins

1.6.1 Structure and basic functions

Integrins are a family of cell surface receptors for extracellular matrix ligands or cell-surface ligands, and mediate mechanical and chemical signal from the cell surface (Giancotti. et al. 1999). Integrins are transmembrane heterodimers which contain two distinct chains- α (alpha) and β (beta) subunits. The integrin family is composed of at least 24 heterodimers assembled from 18 alpha and 8 beta subunits. Integrin subunits span the plasma membrane. Generally, integrins have very short cytoplasmic domains of about 40–70 amino acids. Extracellulary, the alpha and beta chains lie closely together and form a ligand-binding region for the extracellular matrix. Integrins bind to extracellular matrix proteins such as collagen, laminin, and fibronectin. Integrin α1β1, α2β1, α3β1, α6β1, α7β1, and α6β4 have been shown to be laminin receptors (Delwel. et al. 1996).

1.6.2 Integrin signalling pathways

Integrins generate intracellular signals either by binding extracellular ligand (outside-in signalling) or by regulating cytoplasmic signals within the cell (inside-out signalling) (Hynes et al., 2002). Integrin signalling plays an essential role in regulating cell physiology, including morphology, adhesion, survival or apoptosis, shape, polarity, motility, proliferation, and differentiation. It is proposed that several integrins are expressed on the cell surface in an inactive state and require cellular activation with a variety of agonists to acquire the capability to mediate adhesion to their appropriate ligands (Dustin. et al.1991, Shaw. et al. 1990, Shaw. et al. 1993).

Integrins regulate a variety of signalling pathways such as FAK, PI3K, PKB/Akt, and RAS/ERK pathways which are involved in cell proliferation and cell survival. For example, cellular adhesion through integrins results in phosphorylation of FAK then activates PI3K kinase which provides protective signal acting through PKB/Akt to block entry into cell apoptosis (Khwaia et al., 1997). In addition, the action of RAS/ERK pathway has been shown to promote cell cycle progression and crucial for cell proliferation (Marshall et al., 1995). (Figure 1.8)

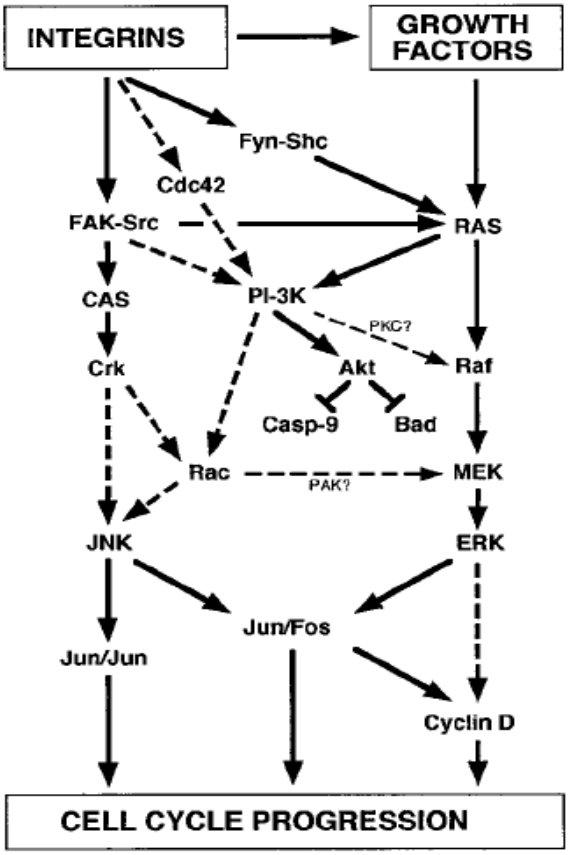


Figure 1.8 Integrin signaling pathway Figure shows major signaling pathways that are known (solid arrows) or presumed (dashed arrows) to be coordinately regulated by integrins and growth factors receptors.

1.7 CD98

1.7.1 Structure and basic function

CD98, a multifunctional membrane protein originally discovered on the surface of activated T cells, is now known to be present in many cell types and all malignant cell lines (Haynes et al., 1981, Deves et al., 2000). CD98 was initially characterized as a T-cell activation marker, and was later shown to function as an amino-acid transporter (Nakamura et al., 1999).

The CD98 family is composed of widely expressed cell-surface disulfide-linked 125-kDa heterodimeric membrane glycoprotein which contains a common glycosylated 80-kDa heavy chain (CD98hc, 4F2hc, SLC3A2) and a group of ~ 45-kDa light chains (Mastroberardino et al., 1998, Hemler et al., 1982) (Fig 1.9). In this heterodimeric form, the CD98 protein transfers specific groups of amino acids across the plasma membrane. The determination of group and mechanism depends on the properties of the specific light chain. Various light chains have the potential to form a complex with heavy chain depending on the cell type and intracellular localization (Nakamura et al., 1999; Dalton. et al., 2007). CD98 has been implicated in a variety of functions including amino acid transport, cell survival, integrin activation, and cell fusion (Warren et al., 1999; Fenczik et al., 1997; Ohgimoto et al., 1995).

1.7.2 The role of CD98 in cell growth and tumorigenesis

It has been shown that CD98 is rapidly up-regulated early in the transition from G0 to G1 phase following cellular activation and remains elevated until the cell cycle is

complete (Azzarone et al., 1985; Suomalainen et al., 1986; Parmacek. et al.1989). In addition, CD98hc is highly expressed on the surface of tumour cells and it is widely believed that CD98hc plays an important role in tumorigenesis (Bellone et al., 1989; Dixon et al., 1990). For example, CD98hc overexpression leads to anchorage-independent cell growth and tumorigenesis (Henderson et al., 2004).

Figure 1. 9

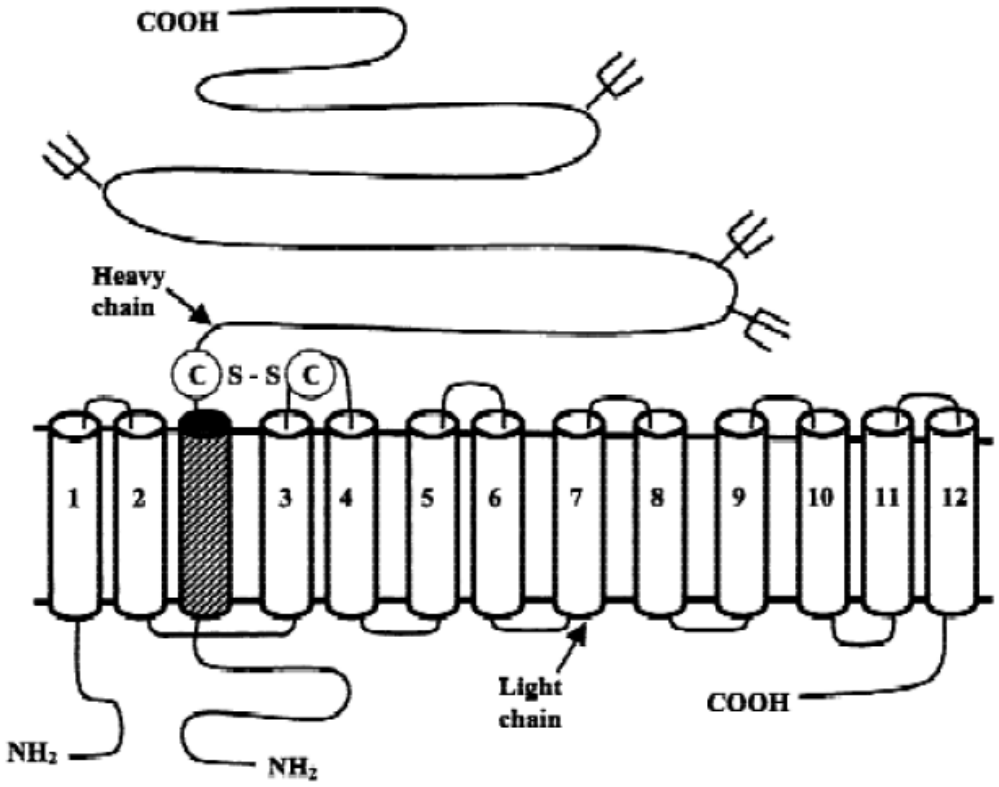


Figure 1.9 Model of CD98 structure. The heterodimer CD98 consist of a glycosylated heavy chain and a group of light chain. The two type of subunits are linked through a disulfide bond. Picture copied from R. Deve´s et al., 1999

1.8 The interactions of galectin-3 with Integrins / CD98

Galectin-3, a proposed ligand for the glycosylated extracellular domain of CD98hc, is one of the candidates of CD98 natural activators (Dalton1et al., 2007). Galectin-3 has been reported to bind integrin α1β1 (Ochieng et al., 1999) and up-regulate integrin expression (Le et al., 1996). Moreover, exogenous galectin-3 can enhance the adhesion of platelets to immobilized fibrinogen or fibronectin by mediating integrin α2β1 or α5β1, respectively (Fujimoto et al., 2000).

Numerous studies have indicated that CD98 is strongly associated with integrin activation. It has been proposed that the cytoplasmic domain of CD98 intracellularly binds to the cytoplasmic tail of specific integrin β-subunits and promotes integrin activation through incompletely identified intracellular signalling pathways (Hughes et al., 2001). For example, integrin-mediated adhesion of human cancer cell lines to fibronectin or laminin was up-regulated by ligation of surface-expressed CD98 with the 4F2 antibody (Fenczik et al., 1997; Chandrasekaran et al., 1999). In addition, the 4F2 antibody also induces T-cell proliferation in a integrin-dependent manner (Warren et al., 2000).

Moreover, several studies indicated that CD98 plays significant roles in regulating integrin-mediated functions such as cell proliferation, survival, adhesion and apoptosis. For examples, CD98 clustering can activate Rap1, a Ras-related GTP-binding protein, and increase the affinity of integrin α5β1 to activate the PI3K signaling pathway (Fujimoto et al., 2000). CD98hc is required for efficient adhesion-induced activation of Akt and Rac which are involved in cell survival and cell migration in ES cells (Feral et al., 2005). In addition, cross-linking CD98 with

4F2 antibody stimulates integrin-mediated increases in focal adhesion kinase (FAK) and PI3K activation (Henderson, 2004).

Galectin-3 has been demonstrated to promote CD98 dimerization and integrin activation. It has been proposed that the association of CD98 and integrin subunits requires dimerization of CD98 and this is mediated by galectin-3. Galectin-3, an endogenous cross-linker of CD98, can ligate the glycosylated extracellular domain of CD98, thus mediate integrin clustering on the surface of cells to increase the avidity of binding (Hughes et al., 2001). This mechanism may mediate cell adhesion to extracellular matrix such as laminin. However, it still remains unclear whether galectin-3 can trigger CD98 mediated-integrin activation (Figure 1.10).

The purpose of my research project was to evaluated the role of galectin-3, CD98, and integrin in regulating LPC induction, proliferation and differentiation which to date not yet been described.

Figure 1. 10

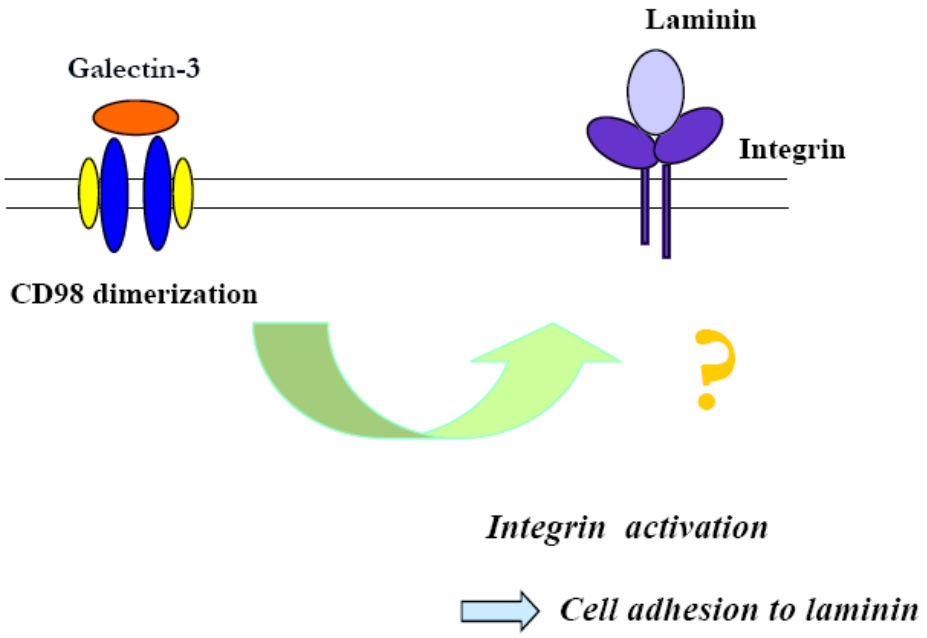


Figure 1.10 Galectin-3 might mediate CD98 dimerization which promotes integrin activation and cell adhesion to laminin. Picture adapted from the review by Hughes,R.C. (2001)

1.9 Aims and hypothesis

In this study I hypothesize that galectin-3 can enhance LPCs adhesion to laminin and plays an important role in regulating LPC induction, proliferation, and differentiation. In addition, integrin activation and CD98 may be involved in regulating LPC behaviour.

To investigate this hypothesis, the following aims were established

1. To investigate the expression of galectin-3 in the livers of CDE-fed mice.

   Firstly, the expression of galectin-3 in the livers of CDE-fed mice was assessed to investigate whether the expression of galectin-3 was strongly associated with LPC activation.

2. To investigate the function of galectin-3 in LPC induction and proliferation *in vivo.*

   The function of galectin-3 in LPC induction was then investigated by using galectin-3 null mice. These mice were put on the CDE diet for 12 days to investigate the role of galectin-3 in LPC induction.

3. To investigate the role of galectin-3 in LPC proliferation and differentiation *in vitro*

   Next, the role of galectin-3 in regulating LPC proliferation and differentiation *in vitro* was assessed by isolating primary LPCs from the livers of the CDE-fed WT and galectin-3 null mice. The expression of galectin-3 on primary LPCs was

analyzed and the proliferation and differentiation assay were then conducted on isolated primary WT and galectin-3 null LPCs.

4. To confirm the role of galectin-3 in LPC differentiation *in vitro* by knocking down galectin-3 expression.

   The role of galectin-3 in LPC differentiation into both hepatic and biliary lineages was confirmed by knocking down the expression of galectin-3 in LPC line: BMOLs. Galectin-3 siRNA was transfected into BMOLs and the effect of knocking down galectin-3 expression on LPC differentiation into both lineages was then analyzed.

5. To investigate the role of laminin in LPC differentiation *in vitro*

   The role of laminin in LPC differentiation was also investigated by culturing BMOLs on laminin-coated or plastic plates under the condition which promote differentiation.

6. To investigate the role of the extracellular binding activity of galectin-3 in LPC proliferation and adhesion to laminin.

   The extracellular binding activity of galectin-3 was blocked by treating BMOLs with lactose, the competitive inhibitor of galectin-3. Then the effect of lactose on LPC proliferation and adhesion was assessed by the MTT proliferation assay and adhesion assay.

7. To investigate the mechanism of galectin-3 regulating LPC proliferation.

   The role of galectin-3 in regulating the expression of cell cycle regulators and

cell-adhesion mediated signalling pathway was then assessed in galectin-3 null primary LPCs and galectin-3 siRNA transfected BMOLs

8. To investigate the expression of CD98 and integrin β1 in the livers of CDE-fed mice and primary LPCs.

To analyze whether CD98 and integrin β1 are involved in regulating LPC behaviour, their expression were investigated in the livers of CDE-fed WT and galectin-3 null mice and primary LPC culture. However, the precise regulatory roles of CD98 and integrin β1 in LPC induction, proliferation, and differentiation were not addressed in this study.

Chapter 2

Material and Methods

2.1 Galectin-3 null mice

Galectin-3 null mice were generously given by Professor Tariq Sethi. Generation of galectin-3 null mice by gene targeting technology has been described previously. (Colnot et al., 1998) Homologous recombination removes exons II, III, and IV, including the initiating codon in exon II in galectin-3. As controls, age- and sex-matched WT C57/B6 mice were used. All animal work was carried out under procedural and ethical guidelines of the Home Office (UK).

2.2 Animal model of LPC induction: Choline-Deficient, Ethionine-Supplemented (CDE diet)

CDE diet, a rodent model of LPC activation, was used to assess the cellular constituents of the liver tissue niche. In the dietary protocol of LPC induction, age- and sex- matched C57/B6 and galectin-3 null mice were fed a diet composed of powdered choline deficient chow (MP biomed) supplemented with DLethionine (Sigma) at 0.15% in sweeten water: Robson orange juice (to improve animal tolerance to ethionine) for 12 days.

After 12 days, both C57/B6 and galectin-3 null mice were sacrificed and the liver tissues were harvested. The liver tissue was either incubated in 10% of formalin for 6

hours or in Methcarn (6% of Methanol, 1% of Acetic Acid, and 3% of chloroform) overnight. The liver tissues were then washed with 70% ethanol and embedded with paraffin.

2.3 Immunohistochemistry (IHC)

3.5 μ m-thick paraffin embedded sections were dewaxed and rehydrated prior to IHC. The following are different antigen retrieval methods for different antibodies. The sections were microwaved in 10 mM sodium citrate buffer (pH 6.0) for 5 min then incubated with proteinase K at 37℃ for 5 min (12.5 % in PBS) (Sigma, UK) (PanCK). The sections were microwaved in 10 mM sodium citrate buffer (pH 6.0) for 20 min then microwaved in Tris buffer EDTA (PH9.0) for 20 min (CD98, galectin-3). The sections were incubated with trypsin (0.01% in 0.1% $CaCl_2$) at 37℃. for 15 min (VWF). The sections were microwaved in Tris buffer EDTA (PH9.0) for 20 min (Desmin) or 10 min (Integrin β 1). The sections were microwaved in 10 mM sodium citrate buffer (pH 6.0) for 10 min (Ki67). There is no antigen retrieval required for Laminin and F4/80 staining.

After the antigen retrieval, the tissue was washed with PBS for 5 min then incubated with 3% hydrogen peroxide for 10 minutes (Sigma, UK) to block the endogenous peroxidase. After washing with PBS, the liver sections were treated with an avidin/biotin kit (Vector Lab ®) to block all endogenous biotin, biotin receptors, or avidin binding sites present in tissues prior to the addition of the labeled avidin reagent. To block nonspecific binding, the tissue sections were incubated with 20% serum (either normal swine, or rabbit serum) for 30 minutes at room temperature.

The tissue sections were then incubated with specific primary antibodies diluted in serum at appropriate dilution for 1 hr. After washing with PBS, biotinylated secondary antibodies were then incubated for 30 minutes followed by a tertiary layer of avidin/biotin peroxidase (R.T.U Vectastain Kit, Vector Lab.®) for 30 minutes at room temperature then 3,3'diaminobenzidine (DAB; DAKO®) was applied to the sections for 5 minutes. The sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and coverslipped.

For the Desmin staining, the Vector® M.O.M. ™ (Mouse on Mouse) immunodetection kits which are designed specifically to localize mouse primary monoclonal antibodies on mouse tissues were used according to the manufacturer's guideline. After the liver sections were treated with an avidin/biotin kit (Vector Lab ®) for 15 minutes, M.O.M. ™ Mouse Ig Blocking Reagent was incubated with liver sectons for 1 hour. After washing with PBS, the tissue sections were incubated with M.O.M. ™ Diluent for 5 minutes. Desmin antibody was the diluted in M.O.M. ™ Diluent to 1:50 and incubated with the sections for 30 minutes. After washing with PBS, M.O.M. ™ Biotinylated Anti-Mouse IgG Reagent was incubated with sections for 10 minutes. A tertiary layer of avidin/biotin peroxidase (R.T.U Vectastain Kit, Vector Lab.®) was then applied for 30 minutes at room temperature then 3,3'diaminobenzidine (DAB; DAKO®) was incubated with the sections for 5 minutes. The sections were counterstained with hematoxylin, dehydrated, cleared in xylene, and coverslipped. The antibodies used for this study were listed below.

Table 2.1 The primary and secondary antibodies used for this study

Primary antibodies	Host animal	Source (Catalogue number)	Dilution
Anti -Pancytokeratin	Rabbit	DAKO	1:200
Anti -F4/80	Rat	eBioscience	1:300
Anti - Desmin	Mouse	DAKO	1:50
Anti - VWF	Rabbit	DAKO	1:50
Anti- galectin-3	Rat (Fitc-conjugated)	Cedarlan Lab Limit	1:200
Anti- CD98	Goat	Santa Cruz	1:200
Anti- Ki67	Rat	DAKO	1:50
Anti- laminin	Rabbit	DAKO	1:200
Anti- Integrin β 1	Rabbit	DAKO	1:50
Secondary antibodies	**Host animal**	**Source**	**Dilution**
swine anti-rabbit	Swine	DAKO	1:400
Rabbit anti Rat	Rabbit	DAKO	1:400
Rabbit anti - FITC	Rabbit	DAKO	1:4000
Rabbit anti- goat	Rabbit	DAKO	1:400

2.4 Immunofluorescence(IMF)

Immunofluorescence was conducted for the double immunostaining of panCK and Ki67; PanCK and galectin-3; PanCK and CD98. 3.5 μm-thick paraffin embedded sections were dewaxed and rehydrated. For antigen retrieval, the sections were microwaved in 10 mM sodium citrate buffer (pH 6.0) for 10 min. To block nonspecific binding, the tissue sections were incubated with 20% donkey serum for 30 minutes at room temperature. The tissue sections were incubated with specific

primary antibodies (Ki67, galectin-3, or CD98) diluted in donkey serum at appropriate dilution overnight. After washed with PBS Tween (0.1%) three times, the tissue was then incubated with secondary antibodies for 30 minutes at room temperature. The sections were washed with PBS Tween (0.1%) three times then incubated with with 20% donkey or goat serum for 30 min. The sections were then incubated with Anti-PanCK antibody for 1 hr. After washing steps, the sections were incubated with secondary antibodies for 30 minutes at room temperature. After washed with PBS in rocker, the sections were then mounted with vector shield (containing DAPI) and the slide was sealed with nail varnish. The antibodies used for the study were listed below.

Table 2.2 The primary and secondary antibodies used for this study

Primary antibodies	**Host animal**	**Source (Catalogue number)**	**Dilution**
Anti -Pancytokeratin	Rabbit	DAKO	1:200
Anti- galectin-3	Rat (Fitc-conjugated)	Cedarlan Lab Limit	1:200
Anti- CD98	Goat	Santa Cruz	1:200
Anti- Ki67	Rat	DAKO	1:50
Secondary antibodies	**Host animal**	**Source**	**Dilution**
Anti-rabbit Alexa 488	Donkey	Invitrogen	1:250
Anti-rabbit Alexa 568	Goat	Invitrogen	1:250
Anti- goat Alexa 546	Donkey	Invitrogen	1:250
Anti-rat Alexa 488	Donkey	Invitrogen	1:250

2.5 Immunocytochemistry(ICC)

Cultured cells were seeded overnight onto the 12-well plate in normal culture conditions. Primary LPCs were fixed with methanol for 20 min then washed with PBS then blocked with 5% normal goat, or donkey serum in PBS for 30 min. Cells were then incubated with primary antibodies in 1% bovine serum albumin (BSA) in PBS for 1hr. Cells were then washed three times with PBS and incubated with secondary antibodies in PBS in darkness for 30min at RT. After three more washes, cells were mounted with with DAPI (Vector Lab. ®). The antibodies used for the study were listed below.

Table 2.3 The primary and secondary antibodies used for this study

Primary antibodies	**Host animal**	**Source (Catalogue number)**	**Dilution**
Anti-CD29(integrin-β1)	Hamster	BD Science	1:100
Anti -Pancytokeratin	Rabbit	DAKO	1:100
Anti- CD98	Goat	Santa Cruz	1:200
Anti – galectin-3	Rat	Cedarlan Lab Limit	1:100
Secondary antibodies	**Host animal**	**Source**	**Dilution**
Anti-hamster Alexa 568	Goat	Invitrogen	1:250
Anti-rabbit Alexa 488	Donkey	Invitrogen	1:250
Anti-rabbit Alexa 568	Goat	Invitrogen	1:250
Anti- goat Alexa 546	Donkey	Invitrogen	1:250
Anti-rat Alexa 488	Donkey	Invitrogen	1:250

2.6 LPCs counting

2.6.1 PanCK positive LPCs counting

40 consecutive non overlapping high power (x20) fields from each animal were counted. LPCs were recognized by the small (approximately 10μm) oval/cuboidal morphology with high nuclear to cytoplasmic ratio. Cells with hepatocyte like morphology (larger (over 20μm) with low nuclear to cytoplasmic ratio) were not counted even if they have weak staining for panCK. Interlobular bile ducts defined as cells small panCK positive cells directly abutting a lumen were excluded.

2.6.2 Ki67 and PanCK double positive LPCs counting

10 non overlapping high power (x20) field pictures from each animal were taken. Ki67 (Red) nuclear positive cells with PanCK membrane positive (Green) cells were counted. PanCK positive (Green) cells with blue Dapi nuclear staining were also counted. The ratio of Ki67 and PanCK double positive cells to PanCK positive cells was then calculated.

2.7 Primary LPC (Liver Progenitor Cells) isolation and culture.

LPCs were isolated from WT and galectin-3 null mice. 5 weeks old mice were treated with the CDE diet plus 0.15% ethionine in sweetened water for 12 days. The liver was chopped into small pieces then incubated with L15 Medium (Sigma), 50ug/ml DNase 1 (Roche), and 250ug/ml Collagenase typeV (Sigma) at 37 ℃ in shaking incubator for 45 min. Liver cells were then strained through a 40um filter

into a 50ml falcon followed by three times of spinning at 50g for 1 min to pellet out hepatocytes and large clumps of cells. After lysing haemocytes with lysis buffer on ice for 5 min, cells were then resuspended in LPC Complete Medium: 45% DMEM High Glucose (PAA), 45% Ham's F10 Medium (PAA) 10% FBS (Hyclone), 1ug/ml Insulin (sigma), 50ug/ml Hydrocortisone (Sigma), 50ug/ml Gentamycin (PAA), 2.5 ml Sodium Pyruvate (PAA). LPCs were then purified by centrifugation through a discontinuous gradient of 20 and 50% Percoll™ (Sigma) in PBS at 1400 × g for 20 min at 4℃. The lower layer was collected separately and washed twice with PBS. Cells were then resuspended in 1X BD imag buffer (BD). Fc Blocks (0.25ug/10^6 cells) was then incubated with cells for 15 min on ice. Cells were then incubated with Rat anti Mouse CD45R/B220 (BD, 50ul/10^7 cells) at 6~12℃ for 30 min. After bring the volume up to 1-8 x 10^7 cells/ml with 1x BD imag buffer, tube was then immediately placed on magnet for 6-8 minutes. Supernatant which contained the $CD45^-$ cells were then collected. The positive fraction off the side of the tube was then washed with 1ml of 1X BD imag buffer and the tube was replaced on the magnet for 5 min. The negative fraction was collected again and then washed with PBS and resuspended in LPC Medium with 10% serum. To reduce fibroblast contamination, cells were gently washed 8–12 h after plating to remove non-adherent cells. Cells were cultured in a humidified atmosphere with 95% O2 / 5% CO2 at 37 °C. To reduce endothelial growth in the cultures, LPC Medium with 5% serum was the replaced after 2-3 days.

2.8 BMOL (Bipotential Murine Lliver Progenitor Cells)

BMOL cell line was generously given by Dr. Belinda Knight. They were established from primary LPC cultures which were isolated from the liver of CDE treated mice

using a technique called “plate and wait” (Strick-Marchand and Weiss, 2002).

Primary LPC cultures were maintained in growth-promoting conditions for several weeks until the cultures reached senescence and the majority of cells died and lifted off the plate. A small number of surviving epithelioid cells formed small colonies and these colonies were expanded, replated at low density and clonally derived cell lines established. Cells were passaged weekly until the cell lines became stable, displaying consistent morphology and growth.(Tirnitz-Parker et al.,2007)

BMOLs were cultured with 5% FBS (PAA), 2mM L-glutamine (PAA), 10U/ml Penicillin; 100ug/ml Streptomycin(PAA), 30ng/ml IGF2 (Invitrogen), 20ng/ml EGF (Sigma) and 10ug/ml insulin (Sigma). Cells were cultured in a humidified atmosphere with 95% O2 / 5% CO2 at 37 °C

2.9 LPC differentiation in vitro

For primary LPCs differentiation, 1ml matrigel (R&D) was thawed at 4°C overnight then suspended in 50 ml LPC Medium supplement with 10% FBS to get homogeneous 2% Matrigel medium. 2% matrigel medium which was supplemented with 50 ng/ml epithelial growth factor (EGF) (Sigma) was then added to the cells. After 1, 3, 5 days of induction, cells were fixed with methanol for staining or the total RNA was extracted from the cells.

For BMOL cell differentiation, 1ml matrigel (R&D) was thawed at 4°C overnight then suspended in 50 ml BMOL cell Medium supplement with 10% FBS (without IGF2) to get homogeneous 2% Matrigel medium. 2% matrigel medium which was

supplemented with 50ng/ml EGF (Sigma) and 30ng/ml human recombinant oncostatin M (OSM) (Roche) was then added to the cells. After 1, 3, 5 days of induction, cells were fixed with methanol for staining or the total RNA was extracted from the cells.

2.10 siRNA transfection

Transfection with Hiperfect (Qiagen) was performed according to the manufactures's protocol. The day before transfection, 1 x 10^5 / well cells were seeded in a 12-well plate in 0.1 ml of BMOL culture medium containing serum and antibiotics and cultured under normal growth conditions. 75 ng of galectin-3 siRNA was added in 100 µl culture medium without serum (this will give a final siRNA concentration of 10 nM) and the reagent was mixed by vortexing. 6 µl of HiPerFect was then added in Transfection Reagent to the diluted siRNA and the reagent was mixed by pipetting up and down 5 times. Transfection Reagent was then incubated for 5–10 min at room temperature (15–25°C) to allow formation of transfection complexes. The complexes were then added drop-wise onto the cells. The plate was gently swirled to ensure uniform distribution of the transfection complexes. Cells were incubated with the transfection complexes under their normal growth conditions. After 3 h, 400 µl of BMOL culture medium was added into cells.

Four siRNA against mouse galectin-3 and scrambled siRNA use as a negative control were purchased from Qiagen. Upon receipt 10nmol lyophilized siRNA was resuspended in 100 μl sterile, RNase-free water to each tube to obtaina 10 μM solution. The followings are target sequences for the four different galectin-3 siRNA.

Table 2.4 The target sequence for four galectin-3 siRNA used for this study

siRNA	Target sequence
Mm_Lgal3_1 (sequence 1)	AACTATGTAATTATCAATAAA
Mm_Lgal3_2 (sequence 2)	CAGGAGAGTCATTGTGTGTAA
Mm_Lgal3_3(sequence 3)	CACAATCATGGGCACAGTGAA
Mm_Lgal3_4 (sequence 4)	CTGCTCGTGACTGCTAGGCAA.

2.11 Quantitative real-time RT-PCR analysis

2.11.1 Total RNA extraction

Total RNA from cultured cells was extracted using RNeasy ® Mini Kit (Qiagen) according to manufacturer's instructions. For cell lines: The cell culture medium was removed and rinsed by PBS. 250 µl of RLT buffer was added to a culture dish and the cells were harvested by cell scraper (Corning) and transferred into a QIAshredder spin column (Qiagen), followed by centrifugation at 13,000 rpm for 2 minutes. An equal volume of 70% ethanol was added to the homogenized lysate. After mixing well by pipetting, the mixture was transferred to an RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. 700 µl of buffer RW1 was added to the column and the column was centrifuged for 15 seconds. Next the column was washed twice with 500 µl of RPE buffer, followed by centrifuging for 1 minute. Finally, 30 µl of the RNase-free water was added to elute the RNA. The concentration of RNA was then determined by NanoDrop. (Thermo)

For extracting RNA from the liver tissue, less than 30 mg of liver tissue was placed

into 1.5 ml eppendorf. The tissues was disrupted and homogenized by the vessel in RLT buffer containing ß-ME and then the mixture was transferred into a QIAshredder spin column, following by centrifugation at 13,000 rpm for 2 minutes. An equal volume of 70% ethanol was added to the homogenized lysate. After mixing well by pipetting, 700 µl of the mixture was transferred to an RNeasy spin colum and centrifuged for 15 seconds at 10,000 rpm. 700 µl of buffer RW1 was added to the column and the column was centrifuged for 15 seconds. Next the column was washed twice with 500 µl of RPE buffer, followed by centrifuging for 1 minute. Finally, 30 µl of the RNase-free water was added to elute the RNA. The concentration of RNA was then determined by NanoDrop. (Thermo)

2.11.2 Reverse Transcription

Riverse Transcription was performed by using QuantiTect Rev. Transcription Kit (Qiagen) in 20 µl reactions. RNase inhibitor and dNTPs are already included in the kit components. gDNA Wipeout Buffer was used to eliminate Genomic DNA contamination in RNA samples. The following represent one reverse transcription reaction:

One Reaction mix:

RNA	1 µg
gDNA Wipeout Buffer	2 µl
RNAse-free water	variable
Total volume	14 µl

This mixture was incubated at 42˚C for 2 minutes to eliminate Genomic DNA contamination in RNA samples and then placed on ice for over 1 minute. Then the following reaction mix was prepared and added previous mixture .

One Reaction mix:

5X Quantiscript RT Buffer (contains Mg2+ and dNTPs)	4 µl
RT Primer Mix	1 µl
Quantiscript Reverse Transcriptase	1 µl

The total 20 µl of mixture was mixed by pipetting gently and then incubated at 50˚C for 30 minutes and then 95˚C for 3 minutes to inactivate Quantiscript Reverse Transcriptase. The cDNA was then ready to serve as template for quantitative PCR (qPCR) reaction.

2.11.3 Quantification of mRNA using real-time PCR

qPCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen) QuantiTect Primers used in qPCR reactions were purchased from Qiagen (Table 2.5) The following volumes represent one PCR reaction:

1x Reaction mix

2x Quantifast SYBR Green PCR master mix	12.5 µl
10x QuantiTect Primers	2.5 µl
cDNA templates (dilute 1 in 200 from 1µg of cDNA)	10 µl
Total volume	25 µl

Reaction was then transferred into MicroAmp® Optical 96-Well Reaction Plate (Applied Biosystems). qPCR reactions were performed by the following steps: Pre-incubation at 95˚C for 5 minutes for PCR initial activation step, denaturation at 95˚C for 10 seconds, combined annealing and extension at 60˚C for 30 seconds, the last two steps were repeated for 35 cycles. Primers were purchased from Qiagen. The

following primers were used for SYBR Green real-time RT-PCR

Table 2.5 The primers used for SYBR Green real-time RT-PCR

Gene	Catalog number
Peptidylprolyl isomerase A:	Mm_PPIA_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00247709)
Albumin	Mm_Alb_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00115570)
Aquaporin-1	Mm_Aqp1_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00109242)
Gamma-glutamyl transpeptidase(GGT)	Mm_Ggt1_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00104209)
Cytokeratin 19(CK19)	Mm_Krt19_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00156667)
Alpha-fetoprotein (AFP)	Mm_Afp_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00174020)
Cytokeratin 7(CK7):	Mm_Krt7_1_SG QuantiTect Primer Assay Qiagen (Cat No. QT00173649
Delta-like 1 (DLK 1)	Mm_Dlk1_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00134344)
Hepatocyte Nuclear Factor 4(HNF4)	Mm_Hnf4a_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00144739)
Galectin-3	Mm_Lgals3_1_SG QuantiTect Primer Assay Qiagen (Cat. No. QT00152558)

2.12 Sodium dodecedyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blotting

2.12.1 Preparation of protein from cells

Medium was removed and the cells were rinsed with ice cold PBS. 100 µl of ice cold lysis buffer with protease inhibitors (1:100 PMSF, 1:500 Aprotinin, 1:500 Levpeptin, 1:100 Benzunidine, and 1:100 Orthovaradate) was added into a well of 12 well plate (35 mm culture dish) and cells were scraped on ice and the cell lysate was transferred to a 1.5 ml eppendorf. The cell lysate was then sonicated 5 times (2 seconds sonication with 5 second rest intervals). To remove the debris, the tube was centrifuged at 13,000 rpm for 10 minutes at 4˚C, and the supernatant was collected and stored at -20˚C until required.

2.12.2 Bradford assay and protein sample preparation.

Protein content was quantified by using Bradford assay. The sequential dilution of the standards was prepared as 5, 2.5, 1.25, 0.625, 0.3125, 0.15625, 0.078125 µg/µl of BSA. 5µl of each dilution of the standard and protein samples was added into a 96 well plate, in triplicate. The Bradford reagent was prepared (75µl H2O + 75µl Bradford for each sample) and 150 µl prepared mixture was then added to each wells. The plate was then read at 595 nm on microplate reader. The figure of the concentration of protein standards vs. OD was the plotted to determine protein concentrations of unknown from the standard curve. 10 µg of protein was then added with 5x Lemmli and then boiled for 10 minutes at 95˚C for SDS-PAGE gel.

2.12.3 SDS-PAGE gel

The glass plates and gel holder were cleaned thoroughly and the glass plates were inserted into the gel holder. The upper gel was added into glass plates quickly after the lower gel was added. The comb was then placed in and the gel was left for 30 min. Then the gel was placed in the holder and 1x running buffer was filled up. The individual well was washed by needles. Both standards and samples were loaded and then gel was run at 90V until the samples reached the end of gel.

2.12.4 Transfer Blotting

The SDS-PAGE gel was assembled in the transfer cassette with the order of (negative anode pole) - sponge / Whatman blotting paper / PAGE / nitrocellulose membrane (Whatman) / Whatman blotting paper / sponge - (positive cathode pole). The transfer cassette was placed in a transfer tank with transfer buffer and the transfer was performed at 300 mA for 1hours at 4˚C.

After transfer, the nitrocellulose membrane was left air dry for 30 min then stained by Ponceau-S (0.2% Ponceau-S in 3% trichloroacetic acid) to check the efficiency of transfer. The membrane was then washed by TBST several times and blocked in 5% skimmed milk powder solution (prepared in TBST) for 1 hour at room temperature. The membrane was incubated with antibodies at appropriate dilutions (in 5% milk or 5% BSA in TBST) for 1 hour at room temperature or overnight at 4˚C. To remove excess antibody, the membrane was washed by TBST for 3 times, 5 minutes each. The membrane was incubated with horseradish peroxidase-linked (HRP) secondary

antibody (1:2000 in 5% skimmed milk or BSA / TBST) for 1 hr at RT. The immune-signal was detected by using chemiluminescent HRP substrate kit (Millipore) according to the manufacturer's instructions (GE Healthcare, RPN2106) and developed in a Medical Xray Fijifilm (Fisher).

The antibodies used in this study were listed in below table.

Table 2.6 The list of the primary and secondary antibodies

Primary Antibody	Host animal	Source	Dilution
Anti-actin	Rabbit	Santa cruze	1:1000
Anti-phospho-FAK	Rabbit	Cell Signaling	1:1000
Anti-AKT	Rabbit	Cell Signaling	1:1000
Anti-Phospho-AKT	Rabbit	Cell Signaling	1:1000
Anti-Phospho-ERK	Rabbit	Cell Signaling	1:1000
Anti-FAK	Rabbit	Cell Signaling	1:1000
β-catenin	Mouse	Millipore	1:500
Anti-Phospho-GSK3	Rabbit	Cell Signaling	1:1000
Anti-cyclinD	Mouse	Cell Signaling	1:1000
Secondary antibodies	**Host animal**	**Source (Catalogue number)**	**Dilution**
Anti-mouse HRP	Horse	Cell signaling	1:2000
Anti-rabbit HRP	Goat	Cell signaling	1:2000

2.12.5 Stripping nitrocellulose membranes

A nitrocellulose membrane can be re-used for another immunological detection. Previous immune-complexes were removed by incubating the membrane in stripping solution for 15 minutes at 60˚C. The membrane was washed 4 times with TBST, before being used for another probing.

2.12.6 Buffers

Lysis buffer:

50Mm Tris, 80Mm KCl, 10Mm EDTA

5x Lemmli

1.5g TrisBase, 5g SDS, 250mg Bromphenolblue(0.05%), 25ml Glycerol, 5ml Mercaptoethanol, made up to 20ml H20

Lower Gel

Lower gel stock was made from 40ml lower gel buffer (90.8 g Tris Base, 2g SDS, made up to 500ml H2O PH 6.8), 36ml Glycerol, 64ml Acrlamide, and 20ml H20,

Lower gel was made up by 11ml lower gel stock, 60 µl APS and 25 µl TEMED.

Upper Gel

Upper gel stock was made from 50ml upper gel buffer (30g Tris Base, 2g SDS, made up to 500ml H2O PH 9.8) , 32ml Acrlamide and 118ml H20

Upper gel was made up by 6ml upper gel stock 40 µl APS and 20µl TEMED.

10x running buffer:

10g SDS, 144g Glycin 30.3 g Tris Base, made up to 1L H2O

10x Transfer buffer:

25 mM Tris, 192 mM glycine and 20% methanol

TBST :

150 mM NaCl, 20 mM Tris pH7.4, 0.1% Tween-20

Stripping Buffer:

100 mM β-mercaptoethanol, 2% SDS and 50 mM Tris-HCl pH6.7

2.13 MTT Cell Proliferation Assay

Cells were plated (7.5×10^3 cells / well) in septuplicate onto a 96-well cultured plate and treated with 50mM lactose or 50mM sucrose for 2 or 4 days. To measure the cell proliferation, 20 μl of 5 mg/ml MTT (Sigma) was added into each well, including one set of wells with MTT but no cells for control and incubated for 3.5 hours at 37˚C in culture hood. 150 μl of DMSO was then added into wells and the plate was put on the shaker for 5 minutes. Cell viability was expressed as optical density (OD), which was detected in the enzyme-linked immunosorbent assay (ELISA) reader at 590 nm wavelength.

2.14 Cell Adhesion Assay

For BMOL cells, the final cell suspension for the adhesion assay contained 2×10^6 viable cells/ml in serum-free medium. Plate was coated with 10% poly-ly-sine for positive control or laminin for 2 hour at 37˚C then blocked with 1 % BSA for 1hour.

The plate was then washed with PBS. The cell suspensions were preincubated with 50mM lactose or 50mM sucrose for 2 hour prior to plating. To initiate adhesion, 50 µl (1×10^5 cells) per well was plated. Adhesion was allowed to proceed for up to 1 hour at 37 °C/ 5% CO_2. After incubation, the plate was washed twice at room temperature with PBS. The attached cells are then fixed with methanol for 2 min. The methanol was then flicked off, and the plate was incubated with Diff-Quick pink solution for 2 min. The solution was flicked off then the plate was incubated with Diff-Quick blue solution for 2 min. The plate was washed with tap water then 50 μl DMSO was added into wells. The plate was then read in a plate reader at 660 nm wavelength. The average absorbance of ten wells was then obtained.

2.15 ELISA Assay

The quantity of galectin-3 was measured by the Duoset ELISA Development kit (R&D Systems) according to the manufacturer's instruction. 100 μl of capture antibody was diluted to the working concentration (2.0 μg/mL) in PBS and coated on 96-well plate overnight at room temperature. The plate wash then washed with wash buffer three times and blocked with 1% BSA for 1 hour. 100 μl of sample medium and the standard reagents was then added into the plate and incubated for 2 hours at room temperature. The plate wash washed and 100 μl of the detection antibody, diluted in 1% BSA was added to each well and incubated for 2 hours at room temperature. Following washing step 100 μl of the working dilution of Streptavidin-HRP was added to each well and incubated for 20 minutes at room temperature; direct light was avoided. After washing, 100 μl of Substrate Solution was added to each well and incubated for 20 minutes at room temperature; direct

light was avoided. 50 μl of stop solution: 2 N H2SO4 was then added the plate was gently shaked. The optical density was then determined by reading by the plate reader at 450 nm wavelength

2.16 Microscopy and Image Capture

For light microscopy, Axioskop microscope 40 (Carl Zeiss UK Ltd) was used with a Media cybernetics digital camera. Images were analyzed using Qcapture software. Image processing was performed using Adobe Photoshop software (Adobe Systems UK, Uxbridge, Middlesex, UK).

2.17 Statistical analysis

Paired data columns were evaluated using a two-tailed Student's t test with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA)

CHAPTER 3

GALECTIN-3 REGULATES LPC PROLIFERATION AND DIFFERENTIATION

3.1 Abstract

The role of galectin-3 in LPC induction, proliferation, and differentiation were investigated in an experimental model of LPC induction, the CDE diet. Here, the role of galectin-3 in LPC induction and proliferation *in vivo* was investigated by analyzing the livers of CDE-fed galectin-3 null mice. In addition, the role of galectin-3 in LPC proliferation and differentiation *in vitro* were also examined by isolating primary LPCs from the livers of CDE-fed galectin-3 null mice. I identified the important role of galectin-3 in initiating LPC induction and LPC proliferation *in vivo*. Moreover, I demonstrated that galectin-3 is crucial for LPC proliferation but is a negative regulator of LPC differentiation.

3.2 Introduction

Liver progenitor cells (LPCs) respond to hepatic injury when hepatocyte division is impaired in chronic or severe injuries. Numerous studies have been done to characterize LPCs further due to their regenerative role in numerous liver diseases and their great potential as alternative treatments besides liver transplantation. LPCs are intimately surrounded by a complex of cells and molecules referred to as the "LPC niche". This microenvironment composes of myofibroblasts, macrophages and the basement membrane laminin which has been suggested to play an important role in maintaining LPCs in an undifferentiated state within the niche (Lorenzini et al., 2010). Here, I propose that extracellular laminin may interact with proteins expressed on the cell membrane of LPC and modulate LPC proliferation and differentiation.

Galectin -3 is a member of the β-galactoside-binding lectin of 30 kDa family. It is composed of a short NH2-terminal domain, a repeated collagen-like sequence, and a carbohydrate recognition (CRD) domain which can binds to various glycoproteins such as intergrins and ECM (Liu et al., 1990; Hughes et al., 1994). Galectin-3 has been reported to bind integrins and regulate β1 mediated adhesion to ECM (Friedrichs et al., 2008; Saravanan et al., 2009). Galectin-3 is involved in various biological phenomena, including cell growth and proliferation (Moutsatsos et al., 1987; Inohara et al., 1998), adhesion (Kuwabara and Liu, 1996; Inohara and Raz, 1995; Inohara et al., 1996) and cell survival (Yang et al., 1996; Akahani et al., 1997). However, little research about the function of galectin-3 in the physiologic and pathologic processes of the liver has been published (Henderson et al., 2006; Shimonishi et al., 2001; Hsu et al., 1999; Santucci et al., 2000). Galectin-3

expression has been found significantly increased in regenerative nodules of cirrhotic liver tissues and in hepatocellular carcinoma (Hsu et al., 1999). Moreover, galectin-3 has been found to modulate the growth of fibroblasts by interacting with their surface glycoconjugates (Inohara et al., 1998); however, the role of galectin-3 in regulating LPC proliferation and differentiation has never been demonstrated.

Here, I examined the hypothesis that galectin-3 regulates LPC proliferation and differentiation in an experimental model of LPC induction, CDE diet, using mutant mice lacking the gene encoding galectin-3 which were generously given by Professor Sethi.

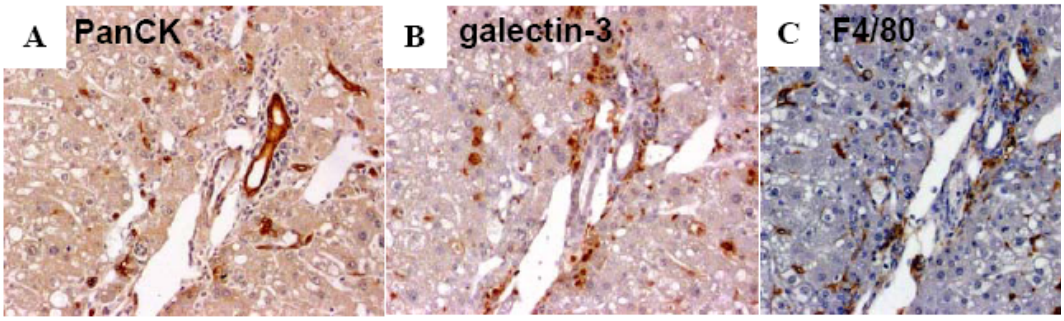
Galectin-3 null mice have been generated by the standard gene targeting method. Homologous recombination removes exons II, III, and IV, including the initiating codon in exon II of galectin-3. The disruption of galectin-3 in the homozygous mutants was confirmed by Southern blot and immunostaining, showing there is no galectin-3 in the homozygous mutants. No overt abnormalities have been shown in these mutant mice with implantation and embryonic development are proceeding normally in galectin-3 null mice (Colnot et al., 1998).

3.3 Results

3.3.1 Galectin-3 expression is strongly associated with the LPC reaction.

WT c57BL/6J mice were put on CDE diet for 12 days to induce chronic liver injury which can cause impaired hepatocyte proliferation followed by activation of LPCs. The expression of galectin-3 was analyzed by immunohistochemistry in the livers of mice that had been given CDE diet for 12 days. LPCs and macrophages were highlighted by the Pancytokeratin (PanCK) and the F4/80 antibody, respectively. Galectin-3 was found strongly expressed adjacent to the PanCK-positive LPCs when LPC reaction was induced by CDE diet. (Fig 3.1A, B, and D) The co-expression of galectin-3 and F4/80 was also observed, indicating galectin-3 was expressed by macrophages. (Fig 3.1B and C) Moreover, the expression of galectin-3 was obviously increased when LPCs were activated by CDE diet, compared to normal mice liver. (Fig. 3.1E) This result was also confirmed by QPCR which showed that CDE-fed mice had significantly higher liver galectin-3 mRNA levels than normal mice. (Fig. 3.1F) These data suggest that LPC activation may be regulated in a paracrine fashion by galectin-3 producing marcrophages.

Figure 3.1



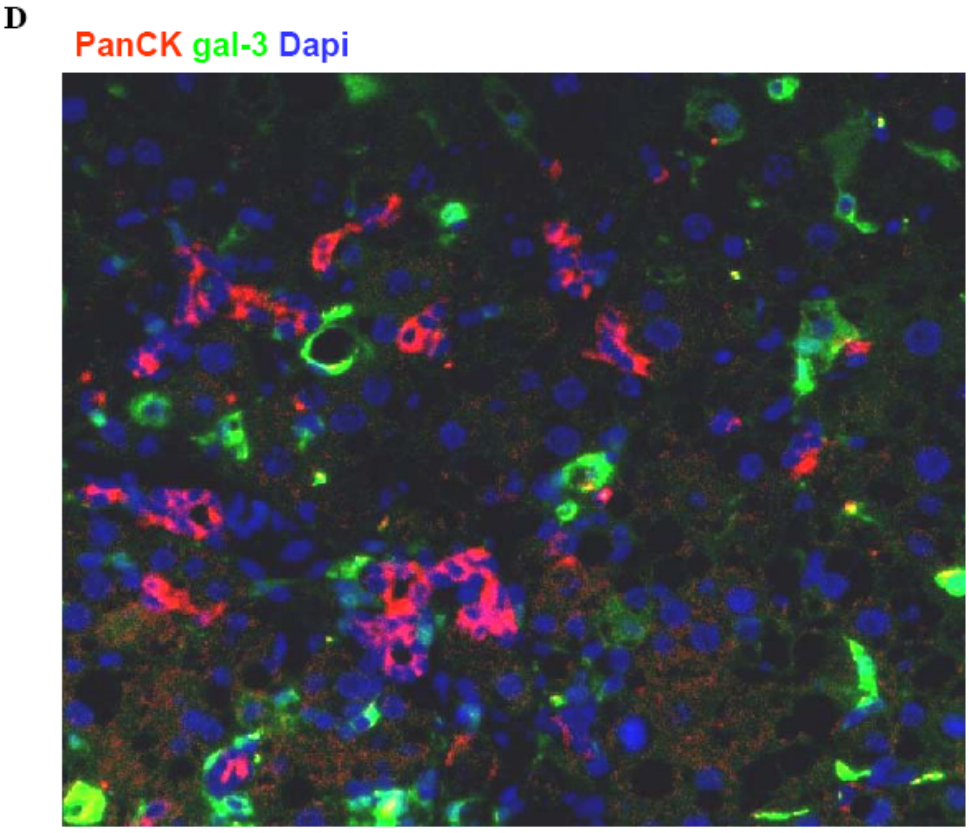
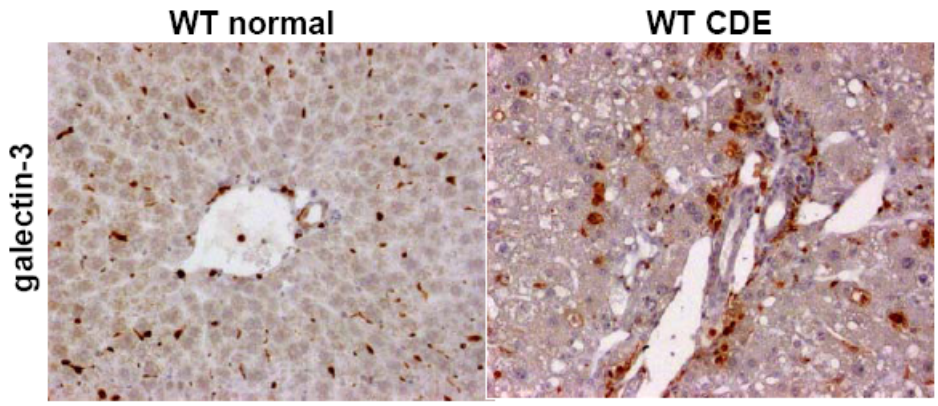


Figure 3.1

E


WT normal
WT CDE
galectin-3

F

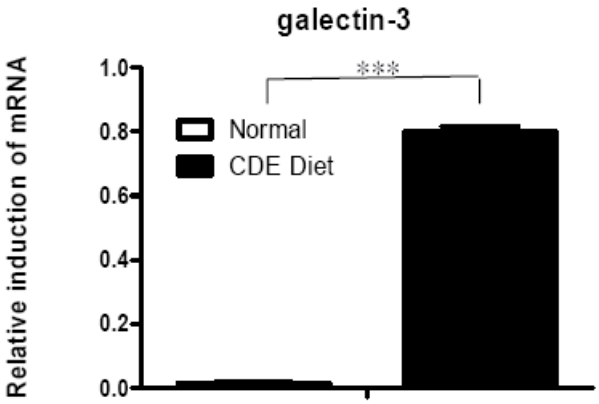

galectin-3
***
Normal
CDE Diet
Relative induction of mRNA
1.0
0.8
0.6
0.4
0.2
0.0

Figure 3.1

Galetin-3 expression is highly associated with LPC reaction. Immunostaining for (A) PanCK (B) galectin-3 (C) F4/80 was performed on serial sections of liver from the CDE-fed mice. (D) Double staining for PanCK and galectin-3 in the livers of CDE-fed mice. (E) Immunostaining for galectin-3 in the livers of normal and CDE-fed mice. (F) Whole liver gene expression of galectin-3 from 12-day control and CDE diet mice. The relative mRNA levels were normalized to the housekeeping gene: PPIA and expressed as mean + SEM; Asterisk denotes a significant deviation from the mean (Student's T-test, *** $p<0.001$) Original magnification: x 200.

3.3.2 Galectin-3 is required for LPCs activation.

To investigate the role of galectin-3 in LPC activation *in vivo*, both WT and galectin-3 null mice were put on CDE diet for 12 days and the livers from these mice were observed. There were few Ki67-positive hepatocytes in the livers of either CDE-fed WT or galectin-3 null mice (Fig 3.2A), confirming hepatocyte proliferation was inhibited by CDE diet. In both normal WT and galectin-3 null mouse liver, no LPC reactions were identified since PanCK-positive LPCs only appeared around biliary ducts and within or very close to the portal tracts. (Fig 3.2B) As expected, WT mice fed a CDE diet showed typical peri-ductular reaction of progenitor cells. (Fig 3.2B) LPCs appeared singly or in irregular strings without lumens, and sometimes appeared to emerge from the biliary ducts. However, CDE-fed galectin-3 null mice showed only rare cells positive for panCK and these were almost exclusively bile ductular cells located in the periportal track. (Fig 3.2B)

The numbers of PanCK-positive LPCs both in the livers of CDE-fed WT and galectin-3 null mice were quantified. Counts of LPCs in WT v.s. galectin-3 null mice confirmed a difference. (1152 ± 127.7 versus 750.9 ± 15.41 per 20x field, $p=0.0076$, $n=8$) (Fig 3.2C)

Importantly, the absence of an LPC response in the livers of CDE-fed galectin-3 null mice was not associated with protection from the illness-inducing effects of the CDE diet on the mice. In contrast, galectin-3 null mice had higher ALT level (Fig 3.2D) which indicated that galectin-3 null mice had a more severe liver injury. These data showed that galectin-3 null mice were susceptible to CDE diet induced liver injury but failed to respond by mounting an appropriate LPC response.

Figure 3.2

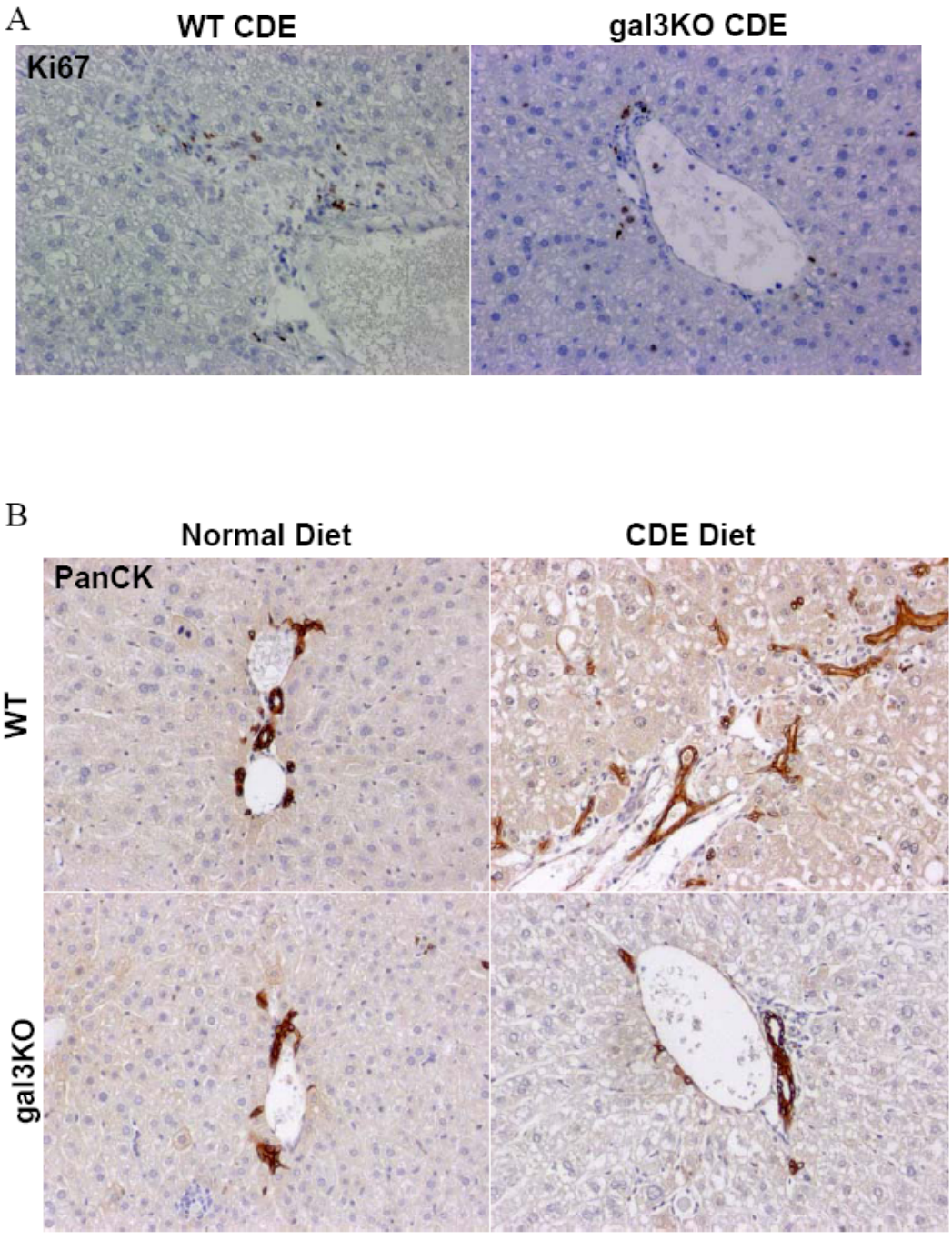
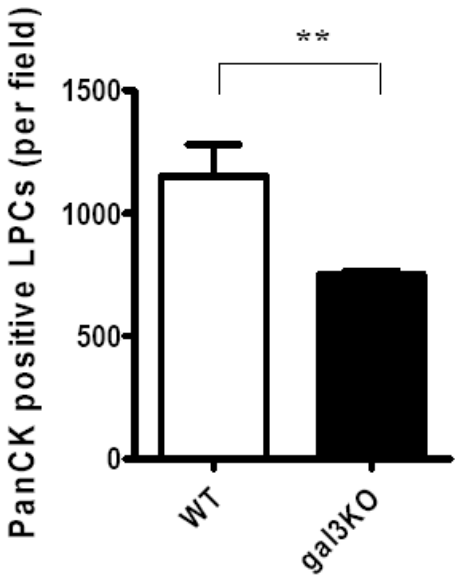

A
WT CDE
gal3KO CDE
Ki67
B
Normal Diet
CDE Diet
PanCK
WT
gal3KO

Figure 3.2

C


**
PanCK positive LPCs (per field)
1500
1000
500
0
WT
gal3KO

D

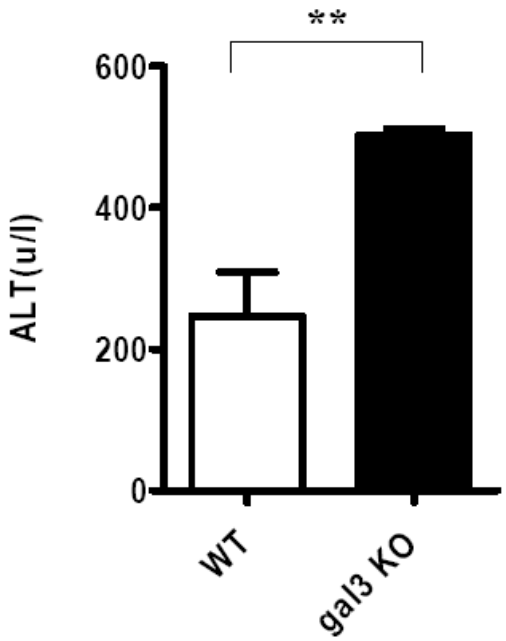

**
ALT(u/l)
600
400
200
0
WT
gal3 KO

Figure 3.2 Galectin-3 null mice did not have observable LPC expansion *in vivo*

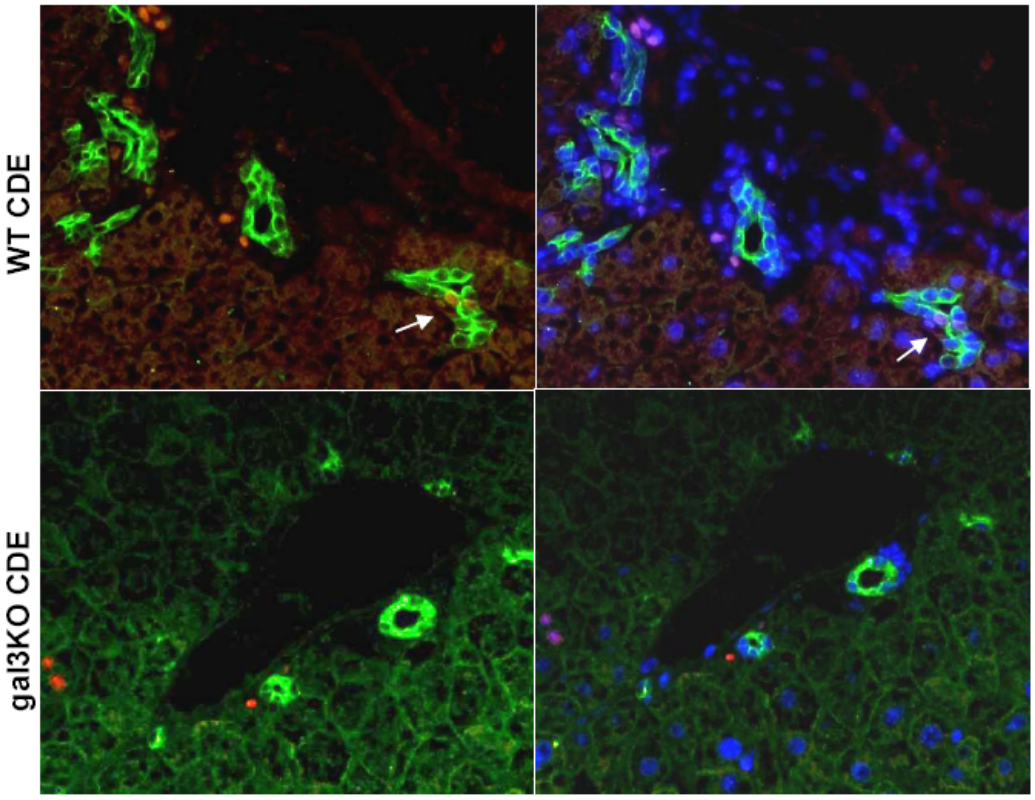
(A) Immunostaining for Ki67 in the livers of CDE-fed WT and galectin-3 null mice (B) Immunostaining for PanCK in the livers of normal WT, galectin-3 null mice and CDE-fed WT and galectin-3 null mice. (C)The number of panCK- positive LPCs in the livers of CDE-fed WT and galectin-3 null mice (1158 versus 751 per 20x field, p=0.0076, n=8) (D) The level of ALT (u/l) in the livers of CDE-fed WT and galectin-3 null mice (246.5 versus 502, p=0.0025, n=5) Data represent mean + SEM; Asterisk denotes a significant deviation from the mean. (Student's T-test, ** P<0.01) Original magnification: x 200.

3.3.3 Galectin-3 is important for LPC proliferation *in vivo*

To address whether the reduced numbers of LPCs in galectin-3 null mice was associated with reduced proliferation of LPCs, double staining for Ki67 and PanCK was conducted in the livers of WT and galectin-3 null mice that had been given CDE diet for 12 days. (Fig 3.3A) Both the numbers of PanCK positive and Ki67/PanCK double positive LPCs were quantified and the percentage of Ki67 positive LPCs was calculated. This analysis showed a highly significant, 7-fold difference between WT and galectin-3 null mice (7.384% V.S. 1.2%, p=0.0072, n=5) (Fig 3.3B), confirming that proliferation of these cells was directly affected by the absence of galectin-3.

Figure 3.3

A


WT CDE
gal3KO CDE
Ki 67PanCK DAPI

B

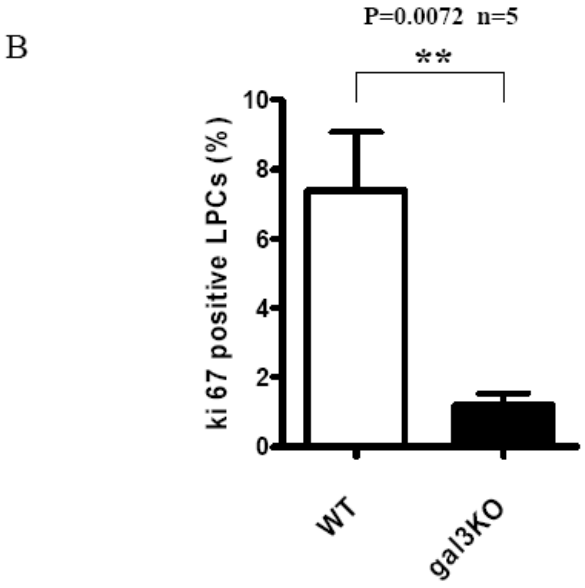

P=0.0072 n=5
**
ki 67 positive LPCs (%)
10
8
6
4
2
0
WT
gal3KO

Figure 3.3 Galectin-3 null mice had reduced LPC proliferation in response to a CDE diet. (A) Double immunostaining for Ki67 and PanCK in the livers of CDE-fed WT and galectin-3 null mice (B) The percentage of Ki67 positive LPCs in the livers of CDE-fed WT and galectin-3 null mice. (7.384% V.S. 1.2%, p=0.0072, n=5) Data represent mean + SEM; Asterisk denotes a significant deviation from the mean. (Student's T-test, ** P<0.01)

3.3.4 Galectin-3 plays an important role in LPC niche formation.

To analyze the LPC niche formation in CDE-fed galectin-3 null mice, immunostaining for PanCK, F4/80 and laminin were performed on serial sections of livers from normal and CDE-fed WT/galectin-3 null mice. Similar expression patterns of F4/80 and laminin were observed in normal WT and galectin-3 null mice. (Fig 3.4A, B) Diffusely spread F4/80 positive macrophages and faint laminin staining were observed in both normal WT and galectin-3 null mouse liver. (Fig 3.4A, B) However, in the CDE-fed galectin-3 null mice macrophages remained diffusely spread throughout the liver whereas in the CDE- fed WT mice hepatic macrophages coalesced around the periportal LPC reactions. (Fig 3.4C) In addition, the expression of laminin was significantly increased in the livers of CDE-fed WT mice, compared to normal mouse liver. (Fig 3.4B, D) Moreover, laminin staining showed the presence of a thicker membrane that spreads from the portal tract and builds a network-like structure intimately surrounding the LPC reaction in the livers of CDE-fed WT mice. (Fig 3.4D) However, the increase in laminin expression and the formation of laminin niche were absent in the livers of CDE-fed galectin-3 null mice. (Fig 3.4D)

Figure 3.4

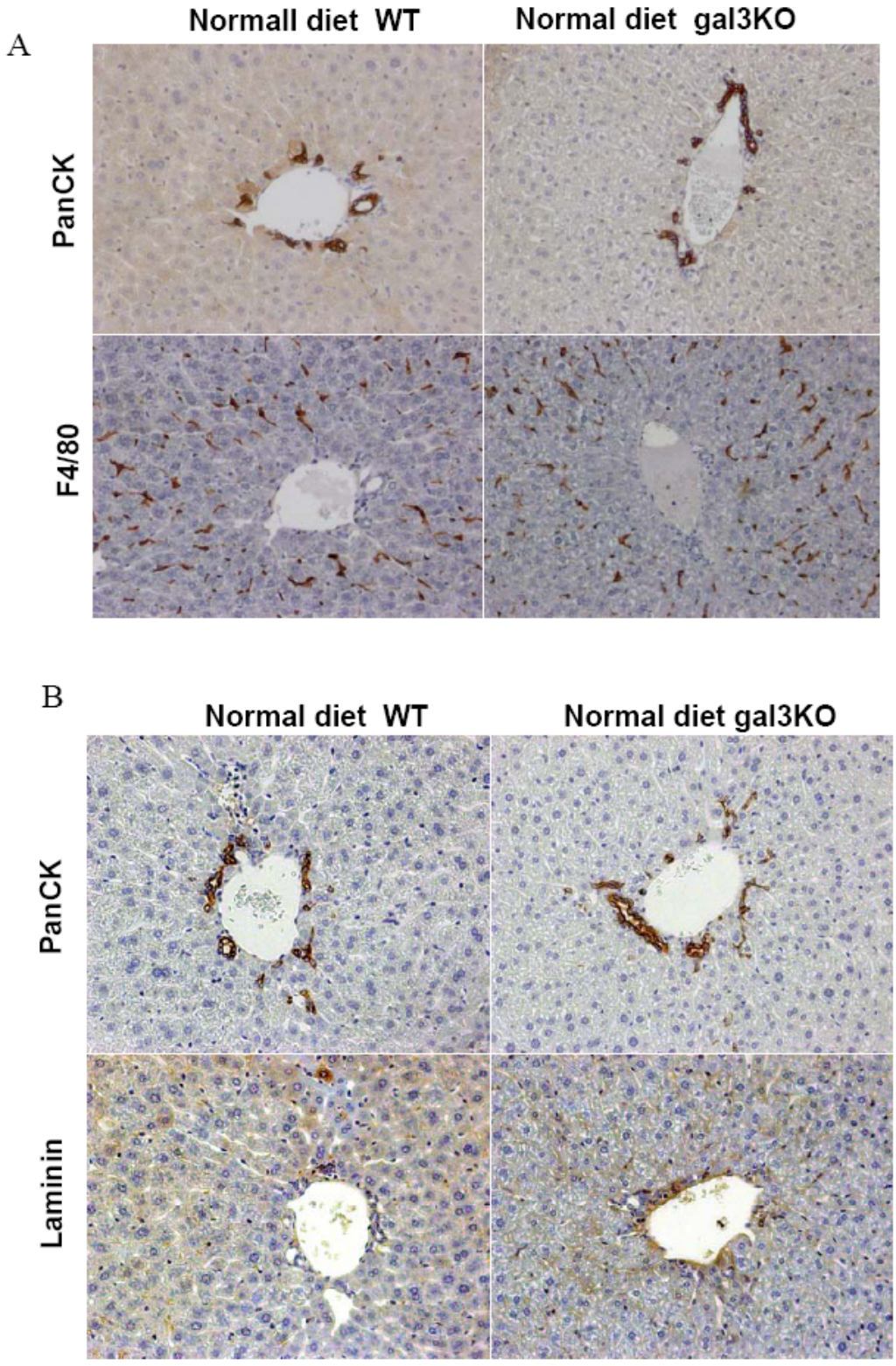

A
Normall diet WT
Normal diet gal3KO
PanCK
F4/80
B
Normal diet WT
Normal diet gal3KO
PanCK
Laminin

Figure 3.4

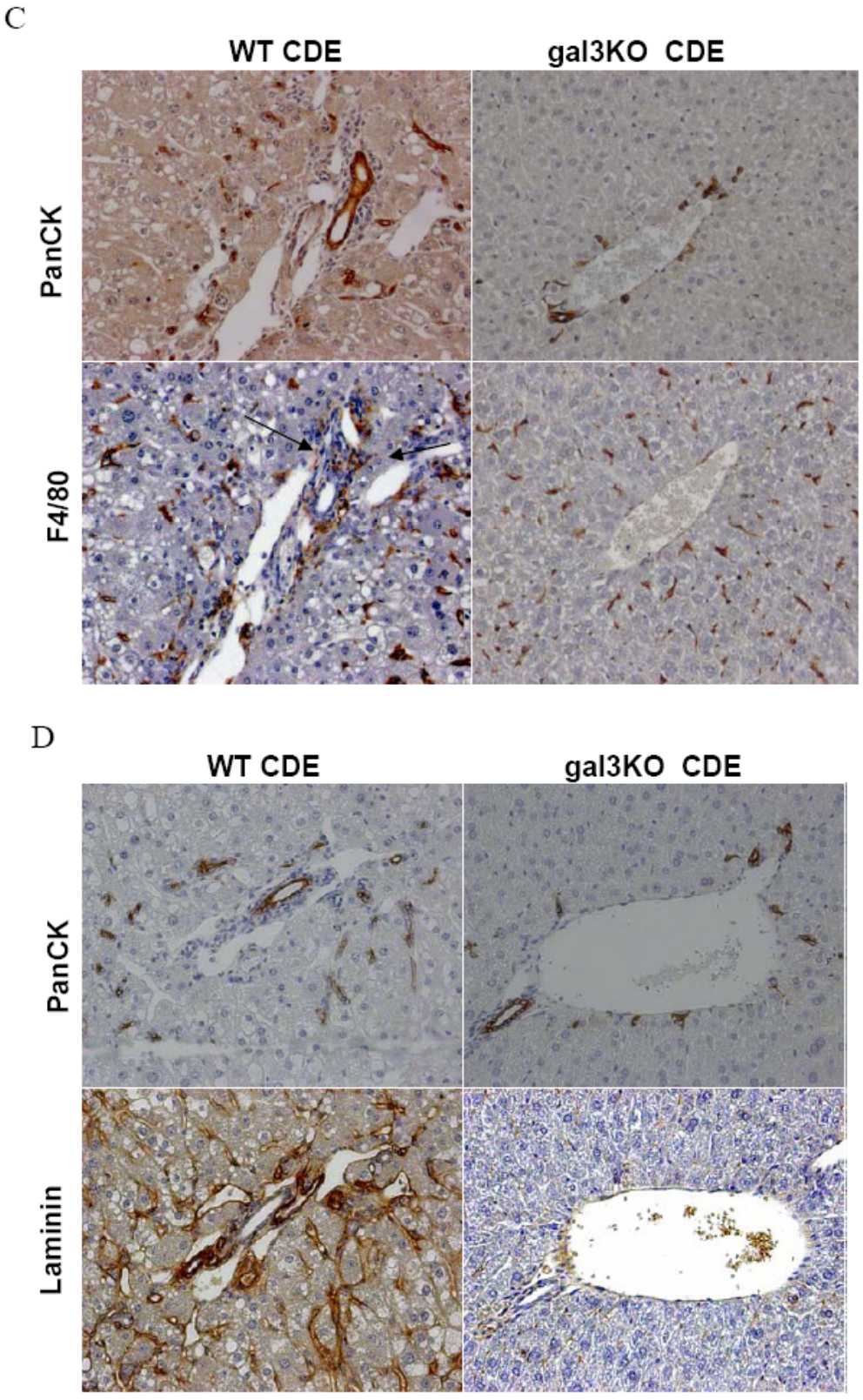

C
WT CDE
gal3KO CDE
PanCK
F4/80
D
WT CDE
gal3KO CDE
PanCK
Laminin

Figure 3.4 Absence of LPC niche formation in the livers of CDE-fed galectin-3 null mice. Immunostaining for (A) PanCK and F4/80 (B) PanCK and laminin on serial sections of livers from normal WT and galectin-3 null mice. Immunostaining for (C) PanCK and F4/80 (D) PanCK and laminin on serial sections of livers from CDE-fed WT and galectin-3 null mice. Original magnification: x 200.

3.3.5 LPCs induction and isolation

Sex- and age- matched WT c57BL/6J and galectin-3 null mice were put on CDE diet for 12 days to induce the activation of LPCs. Primary LPCs were isolated by Percoll gradient centrifugation which has been reported to separate LPCs, hepatocytes, erythrocytes, and debris. Generally, the 50% Percoll™ cell layer mainly consists of hepatocytes and the 20% Percoll™ cell layer consists of LPCs along with cells of similar size and density: primarily inflammatory cells and some other minor cell populations such as myofibroblasts and endothelial cells. (Tirnitz-Parker et al., 2007) Here, we isolated cells from the 20% Percoll™ cell layer. The established cultures were not pure but consisted mainly of LPCs. In order to get a purer population of LPCs, CD45 positive cells (hematopoietic cells) were depleted by using magnetic beads. (Fig 3.5A) Myofibroblasts usually attach to the plates later than LPCs, thus they can be removed by changing medium after 12 hours of culturing the isolated primary LPCs. In addition, the contamination of endothelial cells can be reduced by reducing the concentration of growth serum from 10% to 5% in the culture medium. Here, primary LPCs were successfully derived from the livers of CDE-fed WT and galectin-3 null mice. After seven days of culture either on laminin-coated or plastic plates, cells appeared with oval morphology and a high nuclei/cytoplasm ratio. Around 60% of cells (34.9 ± 5.97 cells/ per 20x field) were panCK positive LPCs by using this method (Fig 3.5B).

Figure 3.5

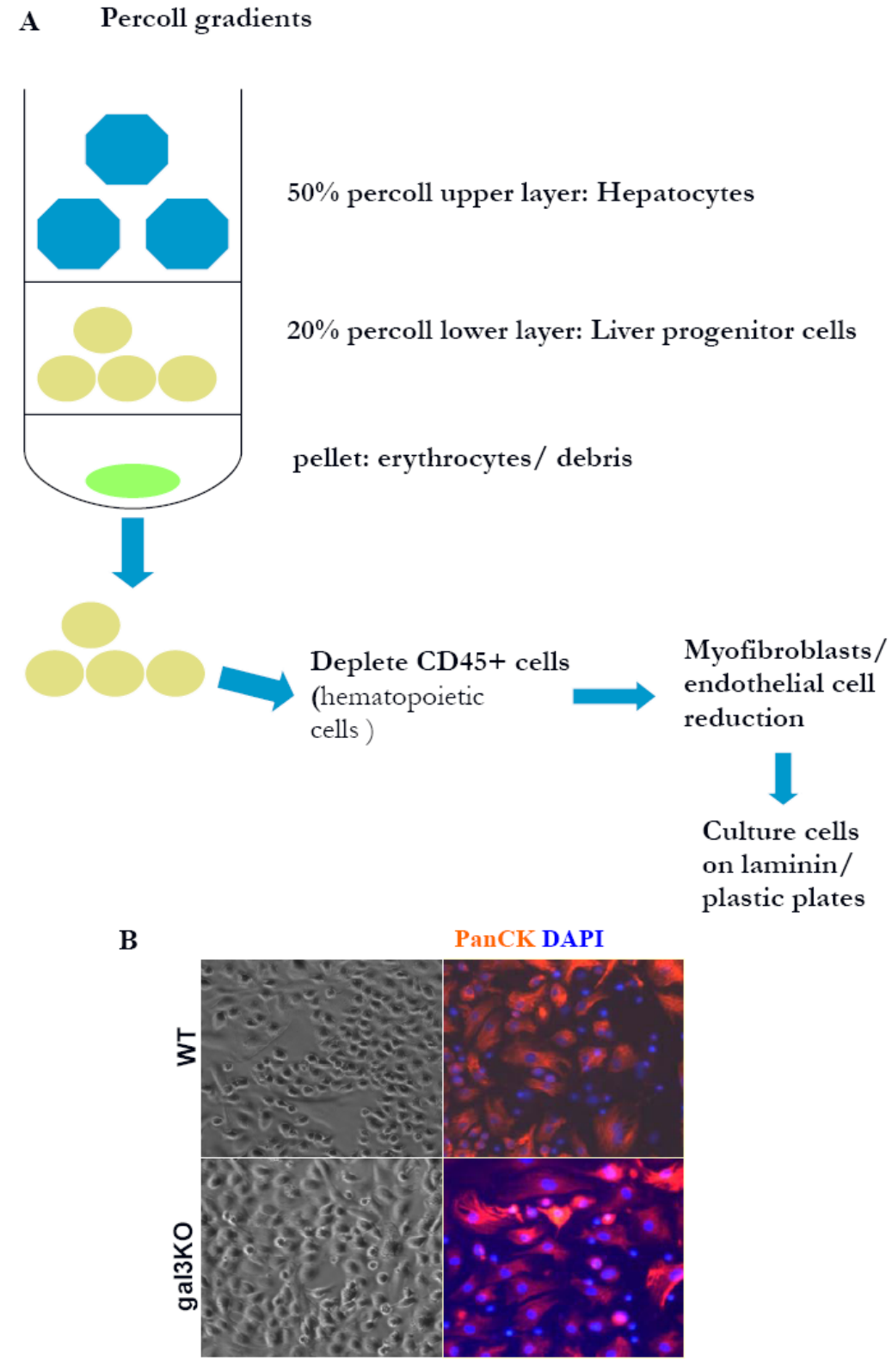

A
Percoll gradients
50% percoll upper layer: Hepatocytes
20% percoll lower layer: Liver progenitor cells
pellet: erythrocytes/ debris
Deplete CD45+ cells (hematopoietic cells)
Myofibroblasts/ endothelial cell reduction
Culture cells on laminin/ plastic plates
B
PanCK DAPI
WT
gal3KO

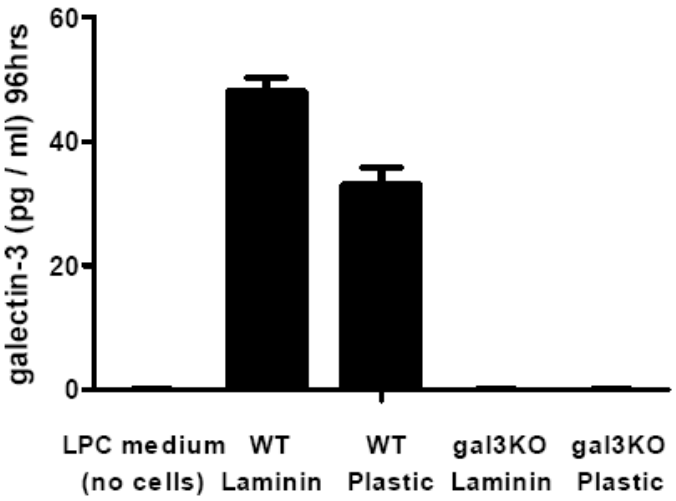
Figure 3.5 LPCs isolation (A) Isolation of primary LPCs from the livers of CDE-fed mice by percoll gradients. (B) Primary LPCs derived from the livers of CDE-fed WT and galectin-3 null mice. Immunostaining for PanCK on primary WT and galectin-3 null LPCs. Original magnification: x 200.

3.3.6 Galectin-3 is expressed and secreted by primary LPCs.

Whether galectin-3 is secreted by primary LPCs extracellularly was assessed. $2x10^5$ per well of primary WT LPCs and galectin-3 null LPCs (negative control) were cultured in a 12 well plate for 4 days then the medium was collected. The quantity of galectin-3 protein level was examined by ELISA. Detection of galectin-3 protein in pure culture medium prior to primary LPCs culturing showed negative. Interestingly, there was around 48pg/ml and 33 pg/ml of galectin-3 in the medium cultured with primary WT LPCs on laminin-coated and plastic plates, indicating that galectin-3 was secreted by primary WT LPCs extracellularly. (Fig 3.6A) Moreover, the expression of galectin-3 on primary LPCs was also analyzed by immunocytochemistry. Expression of galectin-3 was present in a subset of PanCK positive cells and was located through out the cell. (Fig 3.6B)

Figure 3.6

A


galectin-3 (pg / ml) 96hrs
60
40
20
0
LPC medium (no cells)
WT Laminin
WT Plastic
gal3KO Laminin
gal3KO Plastic

B

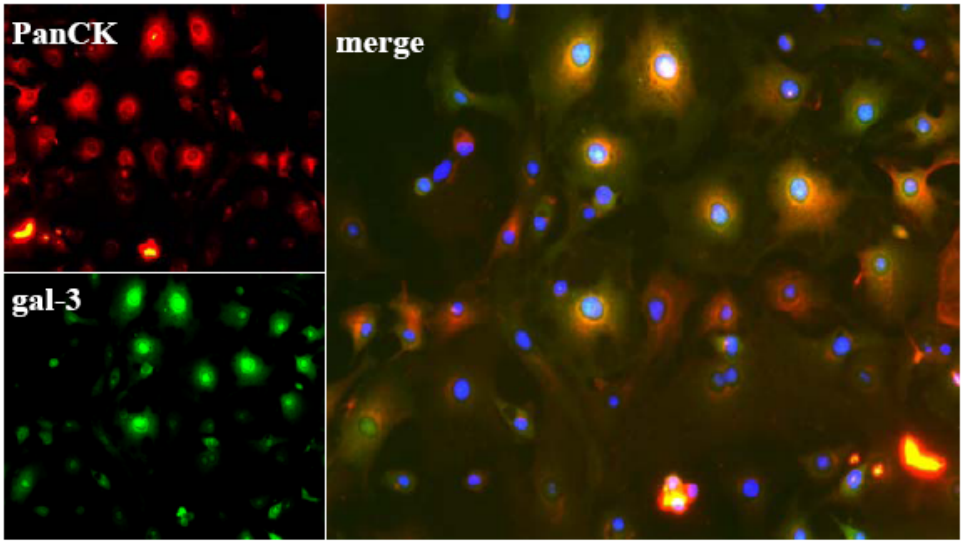

PanCK
gal-3
merge

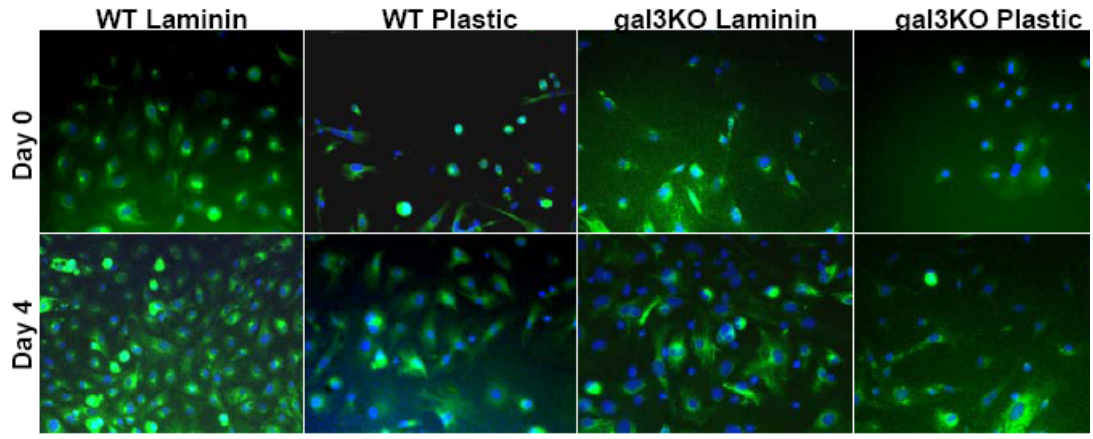
Figure 3.6 Galectin-3 expression on primary WT LPCs. (A) Quantification of Galectin-3 protein level in pure LPC medium and LPC medium collected from culturing $2x10^5$ /well WT and galectin-3 null primary LPCs for 4 days in a 12 well laminin-coated and plastic plate. (B) Double staining for galectin-3 and PanCK on primary WT LPCs. Original magnification: x 200.

3.3.7 Absence of galectin-3 inhibits proliferation of LPCs on laminin

The role of galectin-3 in LPC proliferation *in vitro* was also assessed. After seven days of culture when LPCs exhibited oval morphology with a high nuclei/cytoplasm ratio, both WT and galectin-3 null primary LPCs were cultured on laminin-coated or plastic plates for another 4 days. These cells were then fixed and stained with PanCK antibody. (Fig 3.7A) Ten x20 field pictures were taken and the numbers of PanCK positive LPCs were quantified. After 4 days of culture, WT LPCs cultured on laminin increased significantly compared to galectin-3 null LPCs. (Fig 3.7B) (112.4 versus 58.2 per 20x field, $p < 0.001$) Interestingly, both WT and galectin-3 null LPCs which were cultured on plastic plates did not expand. (Fig 3.7B) This result indicates a requirement of both galectin-3 and laminin in LPC proliferation.

Figure 3.7

A


WT Laminin
WT Plastic
gal3KO Laminin
gal3KO Plastic
Day 0
Day 4

B

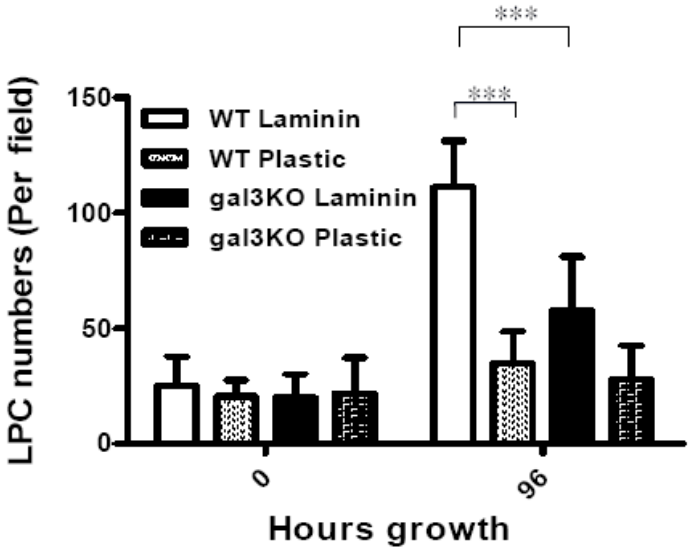

***
***
150
100
50
0
WT Laminin
WT Plastic
gal3KO Laminin
gal3KO Plastic
LPC numbers (Per field)
0
96
Hours growth

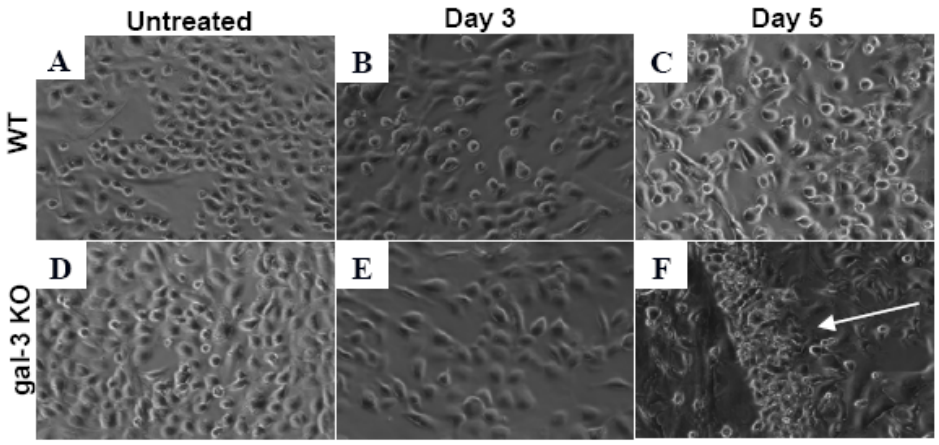
Figure 3.7 Galectin-3 null LPCs did not expand on laminin. (A) PanCK staining on WT and galectin-3 null LPCs which were cultured on plastic or laminin-coated plates at Day 0 and 4. (B) Quantification of PanCK-positive WT and galectin-3 null LPCs per x20 field. Data represent mean + SEM, n=3; Asterisk denotes a significant deviation from the mean (Student's T-test, *** P <0.001) Original magnification: x 200.

3.3.8 Galectin-3 is required to maintain LPCs in an undifferentiated state.

The role of galectin-3 in LPC differentiation *in vitro* was then further analyzed. To assess the role of galectin-3 in LPC differentiation, several different differentiation protocols were attempted to differentiate primary LPCs. Cultured LPCs on matrigel have been shown to favour biliary lineage differentiation (Li, et al., 2002). Moreover, it has been shown that supplementation of the medium with Epithelial growth factor (EGF) can generate hepatocytes (He, et al., 2003). Here, 2% matrigel and 50ng/ml EGF have been shown to differentiate primary LPCs into both biliary and hepatocyte lineages successfully. Both WT and galectin-3 null primary LPC were cultured on laminin and treated with 2 % matrigel and 50ng/ml EGF for 1, 3, and 5 days. (Fig 3.8A-F) Galectin-3 null LPCs, but not WT LPCs formed a confluent monolayer which is similar to hepatic plate –like structure. (Fig 3.8 F, arrow)

RNA was then extracted for quantitative PCR analysis. At time zero, both untreated WT and galectin-3 null LPCs have similar expression of the progenitor cell marker: DLK-1; the biliary cell markers: Aquaporin-1, CK7, and CK19; and the early hepatocyte marker: αFP (Fig 3.8G). After 5 days of differentiation, galectin-3 null LPCs had a higher expression of both the biliary cell markers: CK19, Aquaporin-1, GGT and the hepatocyte markers: αFP and albumin (Fig 3.8H) which indicated that LPCs required galectin-3 to maintain in an undifferentiated phenotype on laminin.

Figure 3.8


Untreated
Day 3
Day 5
A
B
C
WT
D
E
F
gal-3 KO

G

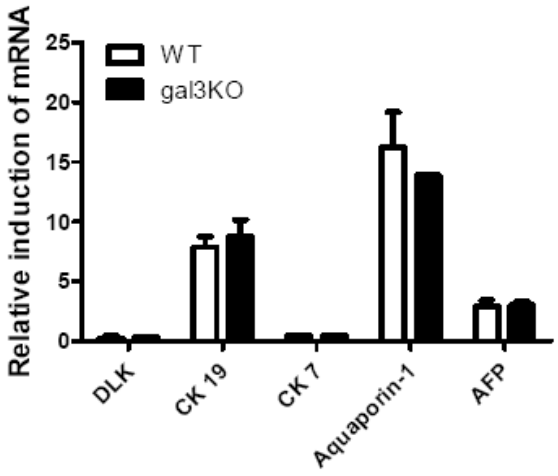

Relative induction of mRNA
25
20
15
10
5
0
WT
gal3KO
DLK
CK 19
CK 7
Aquaporin-1
AFP

Figure 3.8

H

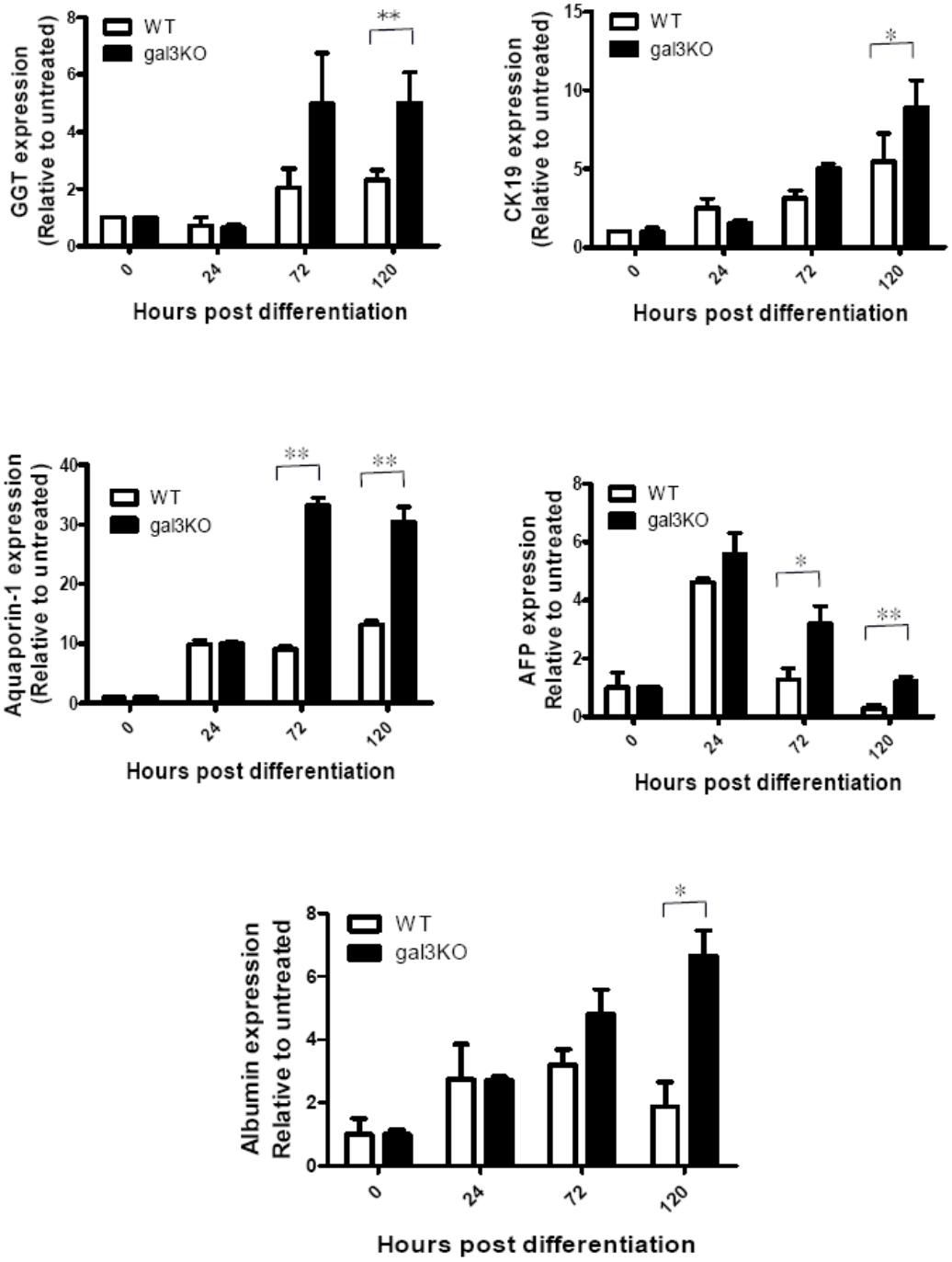

GGT expression (Relative to untreated)
WT
gal3KO
**
Hours post differentiation
CK19 expression (Relative to untreated)
WT
gal3KO
*
Hours post differentiation
Aquaporin-1 expression (Relative to untreated)
WT
gal3KO
**
**
Hours post differentiation
AFP expression Relative to untreated
WT
gal3KO
*
**
Hours post differentiation
Albumin expression Relative to untreated
WT
gal3KO
*
Hours post differentiation

Figure 3.8 Galectin-3 null LPCs differentiated more rapidly

(A-F) The differentiation treatment of 2 % matrigel and 50ng/ml EGF on WT and galectin-3 null LPCs (on laminin-coated plates) for 3 and 5 days. (G) Quantitive PCR analysis of the LPC (DLK-1), the biliary cell (Aquaporin-1, CK7, and CK19), and the early hepatocyte (αFP) markers in undifferentiated WT and galectin-3 null LPCs. The relative mRNA levels were normalized to the housekeeping gene: PPIA (H) Quantitative PCR analysis of the biliary cell (Aquaporin-1, GGT, and CK19) and the hepatocyte (αFP and Albumin) markers in 2% matrigel and 50ng/ml EGF treated WT and galectin-3 null LPCs for 1, 3, and 5 days. The relative mRNA levels were compared with its basal level in untreated WT or galectin-3 null LPCs after being normalized to the housekeeping gene: PPIA. Data represent mean + SEM, n=3; Asterisk denotes a significant deviation from the mean (Student's t-Test, *P<0.05;**P<0.01;***P<0.001) Original magnification: x 200.

3.4 Discussion

Galectin-3 is often associated with inflammation and fibrotic injury in various tissues, including liver. Galectin-3 expression is up-regulated in human and mouse models of liver fibrosis (Henderson et al., 2006) and also in hepatocellular carcinoma (Hsu et al., 1999). Here, I demonstrated that galectin-3 expression was significantly induced when LPCs were activated in the livers of CDE-fed WT mice. In addition, galectin-3 null mice completely lacked an LPC response to the CDE model of liver injury and regeneration, and display showing increased liver injury over WT mice. Not only the total number of LPCs but also the proportion of proliferating LPCs were significantly reduced in galectin-3 null mice, indicating a direct effect on LPC proliferation in the absence of galectin-3.

A previous study has shown that galectin-3 can modulate the growth of fibroblasts by interacting with their surface glycoconjugates (Inohara et al., 1998). In addition, galectin-3 can promote the proliferation of hepatic stellate cells (HSCs) (Naoto et al., 2003). Moreover, the important role of laminin in facilitating LPC proliferation and maintaining LPCs in an undifferentiated state has been suggested (Lorenzini et al., 2010). In this study, I confirmed that plating LPCs on laminin-coated plates facilitates their proliferation. However, galectin-3 null LPCs grew slower than WT LPCs when cultured on laminin-coated plates *in vitro*, suggesting that both laminin and galectin-3 are essential for LPC proliferation. Furthermore, upon treatment with 2 % matrigel and 50ng/ml EGF for 5 days, galectin-3 null LPCs, which were cultured on laminin-coated plates, differentiated into biliary cells and hepatocytes more rapidly than WT LPCs. Taken together, these data suggested that LPCs require galectin-3 to expand on laminin in an undifferentiated state.

In this study, galectin-3 expressing cells were localized to the periportal area of the liver in which the regenerative proliferation of LPC is known to be initiated. In addition, galectin-3 expressing cells were found to co-localize with macrophages which are supporting cells in the LPC niche. Moreover, galectin-3 null mice failed to form the laminin sheaf around LPCs. Also, less recruitment of F4/80+ macrophages to LPCs was observed in galectin-3 null mice, compared to WT mice. This suggested that galectin-3 is important for LPC niche formation. Taken together, these data suggested that galectin-3 may be involved in cross-talk between LPCs and their supporting environment, which is believed to be crucial in regulating LPC proliferation and differentiation.

Laminin has been found to form a dynamic sheath flanking proliferating LPCs and permits LPC proliferation in an undifferentiated form (Lorenzini et al., 2010). Laminin has been shown as one of the counter-receptors for galectin-3 (Ohannesian et al., 1995). In addition, galectin-3 modulates cell adhesion by interacting with integrins or extracelluar matrix such as laminin (Hughes, et al., 2001). Galectin-3 may regulate LPC proliferation by modulating LPC adhesion to laminin. A recent study has shown that galectin-3 can regulate peritoneal B1-cell differentiation into plasma cells (Oliveira et al., 2009). It has been shown that B1 cells tend to arise early from fetal liver-derived precursors and become a self-renewing cell population in the peritoneal cavity of adult mice (Hayakawa et al. 1985; Kantor and Herzenberg 1993; Marcos et al. 1994). The absence of galectin-3, mesenteric membranes are a more adhesive substratum for mononuclear cells which favours differentiation of galectin-3-deficient B1 cells into plasma cells (Oliveira et al., 2009). However, here I hypothesize that galectin-3 enhances LPC adhesion to laminin which favours LPC

proliferation. The absence of galectin-3 results in a weak adhesion to laminin, thus favouring LPC differentiation instead of proliferation. Further research needs to be done to assess the role of galectin-3 in LPC adhesion to laminin which will be demonstrated in the next chapter.

CHAPTER 4

INVESTIGATION OF THE ROLE OF EXTRACELLULAR GALECTIN-3 IN LPC ADHESION AND PROLIFERATION

4.1 Abstract

The role of galectin-3 in initiating LPC activation and maintaining LPCs in a less differentiated state has been demonstrated in the last chapter. The exact mechanism by which galectin-3 regulates LPC behaviour remains unknown. The hypothesis that galectin-3 regulates LPC proliferation by mediating LPC adhesion to laminin was investigated in this chapter. In order to further understand the role of galectin-3 on LPC differentiation, galectin-3 siRNA was transfected into BMOLs which is an established LPC line derived from the livers of CDE-fed mice to knock down galectin-3 expression. In addition, the role of the extracellular binding activity of galectin-3 in LPC proliferation and adhesion to laminin is also discussed in this chapter.

4.2 Introduction

To further analyse the role of galectin-3 in the regulation of LPC proliferation and differentiation, it is essential to have a stable *in vitro* model for the study. A LPC line can be established from bipotential murine liver progenitor cells (BMOLs) from the livers of choline-deficient ethionine- supplemented (CDE)-fed mice (Tirnitz-Parker et al., 2007). The establishment of highly enriched LPC cultures and the derivation of immortalized, non-transformed, clonally derived LPC line from these culture was achieved by using the "plate and wait" method, that was originally developed for the creation of embryonic liver progenitor cell lines (Strick-Marchand et al., 2002). The BMOLs express both hepatic and biliary markers, and have biopotentiality to differentiate in to both hepatic and biliary lineages. In this study, the BMOL line was kindly given by Dr. Belinda Knight for the further analysis of the role of galectin-3 in regulating LPC proliferation and differentiation.

Galectin-3 is not only found in cell cytoplasm or nucleus but also found secreted from cells. Galectin-3 can interact with glycosylated proteins at the cell surface or within the extracellular matrix. There are numerous ligands for galectin-3, including matrix glycoproteins: laminin, collagen IV or fibronectin and cell-adhesion molecules such as integrins. Galectin-3 has also been shown to regulate cell-cell and cell-matrix interaction. In addition, galectin-3 can modulate cell adhesion to extracellular matrix such as laminin by interacting with various β-galactoside containing glycans via its carbohydrate recognition domain (CRD) (Hughes et al., 2001). Galectin-3 may modulate cell adhesion by inhibiting or enhancing cell-cell adhesion and cell interaction with extracellular matrix. In the previous chapter, I

concluded that galectin-3 plays an important role in the maintenance of LPCs in an undifferentiated state. However, how galectin-3 regulates LPC behaviour remains unknown. In this chapter, I hypothesise that galectin-3 regulates LPC proliferation and differentiation by modulating LPC adhesion to laminin. The ability of galectin-3 in enhancing LPCs adhesion to laminin was examined. Firstly, I examined the role of galectin-3 in the BMOL line by transfecting galectin-3 siRNA into BMOLs to knock down galectin-3 expression. Lactose, a disaccharide, has been previously proved to compete with natural ligand recognition by galectin-3 (Platt et al., 1992; Inohara et al., 1994; Pienta et al., 1995). Therefore, it is widely used as a competitive inhibitor of galectin-3. Using a separate approach, BMOLs were treated with lactose to block the extracellular binding activity of galectin-3 to examine its role in LPC adhesion and proliferation.

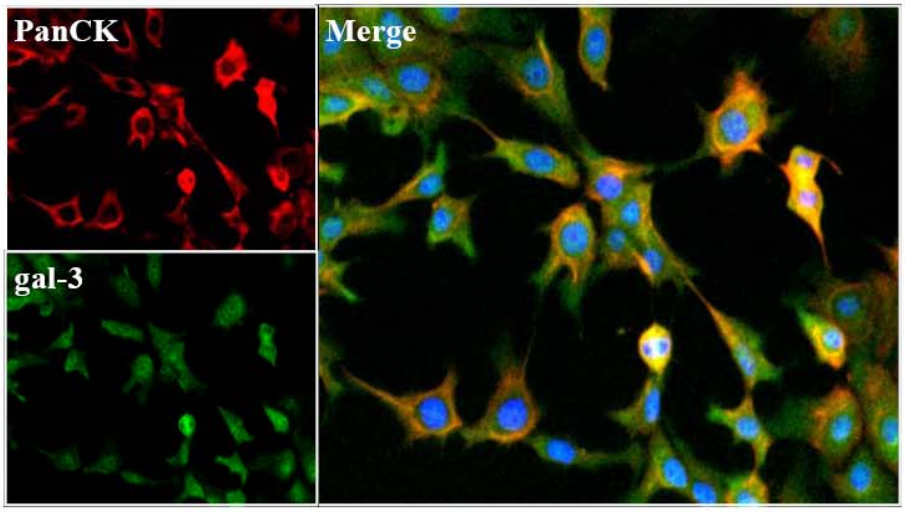
4.3 Result

4.3.1 Galectin-3 is expressed and secreted by BMOLs.

The expression of galectin-3 in BMOLs was examined by immunocytochemistry. Galectin-3 was found expressed by panCK-positive BMOLs. (Fig 4.1A) To analyze whether galectin-3 was secreted by BMOLs extracellularly, $2x10^5$ per well of BMOLs were cultured in a 12 well plate for 4 days then the culture medium was collected. The quantity of galectin-3 protein level in the control culture medium and the medium cultured with BMOLs was examined by ELISA. Here I found that the control culture medium did not contain galectin-3 protein and there was around 100 pg/ml of galectin-3 in the medium cultured with BMOLs, indicating galectin-3 was secreted by BMOLs. (Fig 4.1B)

Figure 4.1

A


PanCK
gal-3
Merge

B

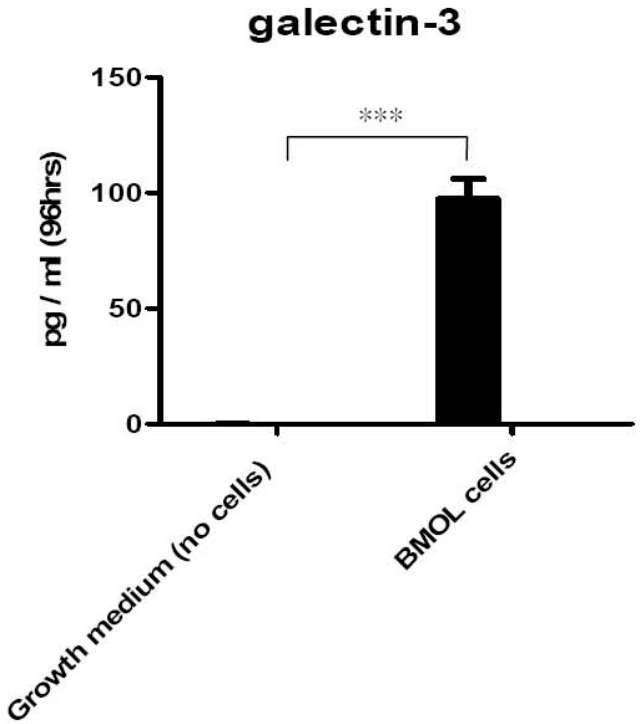

galectin-3
150
100
50
0
pg / ml (96hrs)
***
Growth medium (no cells)
BMOL cells

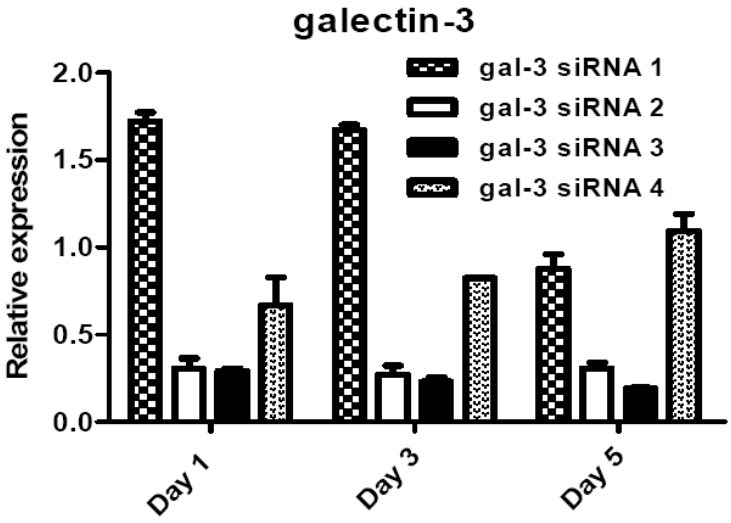
Figure 4.1 Galectin-3 was expressed by BMOLs. (A) Double staining for Galectin-3 and PanCK on BMOLs. (B) Quantification of the galectin-3 protein level in the control culture medium and the medium cultured with $2x10^5$ BMOLs for 4 days. Data represent mean + SEM; Asterisk denotes a significant deviation from the mean (Student's T-test, ***$p<0.001$) Original magnification: x 200.

4.3.2. Successful knock down of galectin-3 expression in BMOLs

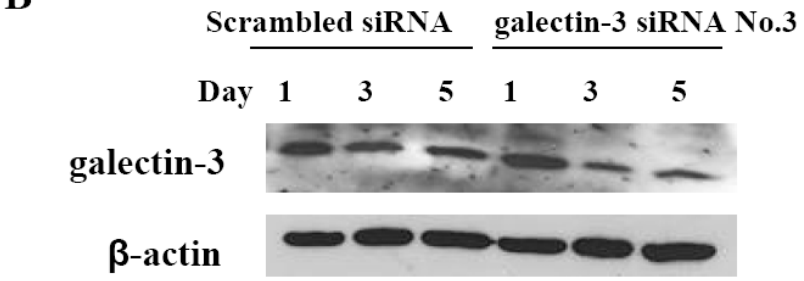
To knock down galectin-3 expression in BMOLs, BMOLs were transfected with four different galectin-3 siRNA and scrambled siRNA as a negative control by using hiperfect for 1, 3, and 5 days. BMOLs were re-transfected with galectin-3 siRNA at day 3 to maintain the knocked down expression level of galectin-3. RNA was then extracted for quantitative PCR analysis. More than 75% of the galectin-3 gene expression was reduced by galectin-3 siRNA No.3. (Fig 4.2A) In addition, the protein expression level of galectin-3 in BMOLs which were transfected by galectin-3 siRNA No.3 for 1, 3, 5 days were also examined to confirm the reduction of galectin-3 expression. (Fig 4.2B) Here, galectin-3 siRNA No.3 was chosen for further experiments.

Figure 4.2

A



B



C

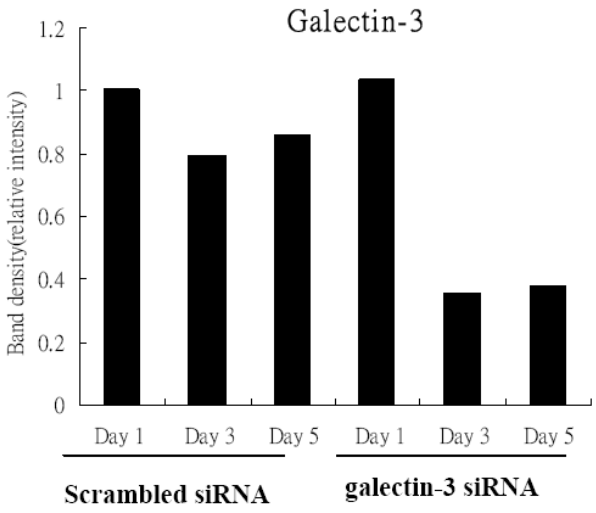


Figure 4.2 Knock down of galectin-3 expression in BMOLs (A) Quantitative PCR analysis of galectin-3 in BMOLs transfected with galectin-3 siRNA No.1~ 4 for 1, 3, and 5 days. The relative mRNA levels were compared with its basal level in BMOLs which were transfected with scrambled siRNA after being normalized to the housekeeping gene: PPIA. (B) Lysates (10μg/lane) of BMOLs which were tranfected with galectin-3 siRNA NO.3 for 1, 3, and 5 days were subjected to immunoblot analysis with an anti- galectin-3, anti- β-actin antibody. (C) Respective densitometry.

4.3.3. Laminin is required to maintain BMOLs in an undifferentiated state.

To confirm the role of laminin in maintaining LPCs in an undifferentiated state, BMOLs were cultured on laminin-coated or plastic plates and differentiated into hepatocyte-like cells and biliary-like cells as follows. Several differentiation protocols were tried to differentiate BMOLs. Oncostatin M (OSM) has been reported as an inducer of hepatocyte-like maturation. (Kamiya et al., 1999; Kamiya et al., 2002; La´zaro et al., 2003) Here, BMOLs were treated with 2% matrigel, 50ng/ml EGF, and 30ng/ml OSM for 3 days to induce differentiation. Only BMOLs cultured on plastic plates but not on laminin-coated plates differentiated into duct-like structures after 3 days of differentiation. (Fig 4.3A, arrow)

RNA was also extracted for quantitative PCR analysis. After 3 days of differentiation, BMOLs cultured on plastic plates had higher gene expression of both the biliary cell markers: CK19, Aquaporin-1, and GGT and the hepatocyte and parenchymal markers: HNF-4α and albumin (Fig 4.3B-F), compared to BMOLs cultured on laminin-coated plates. This result indicates that culturing BMOLs on laminin maintains BMOLs in a less differentiated state.

Figure 4.3

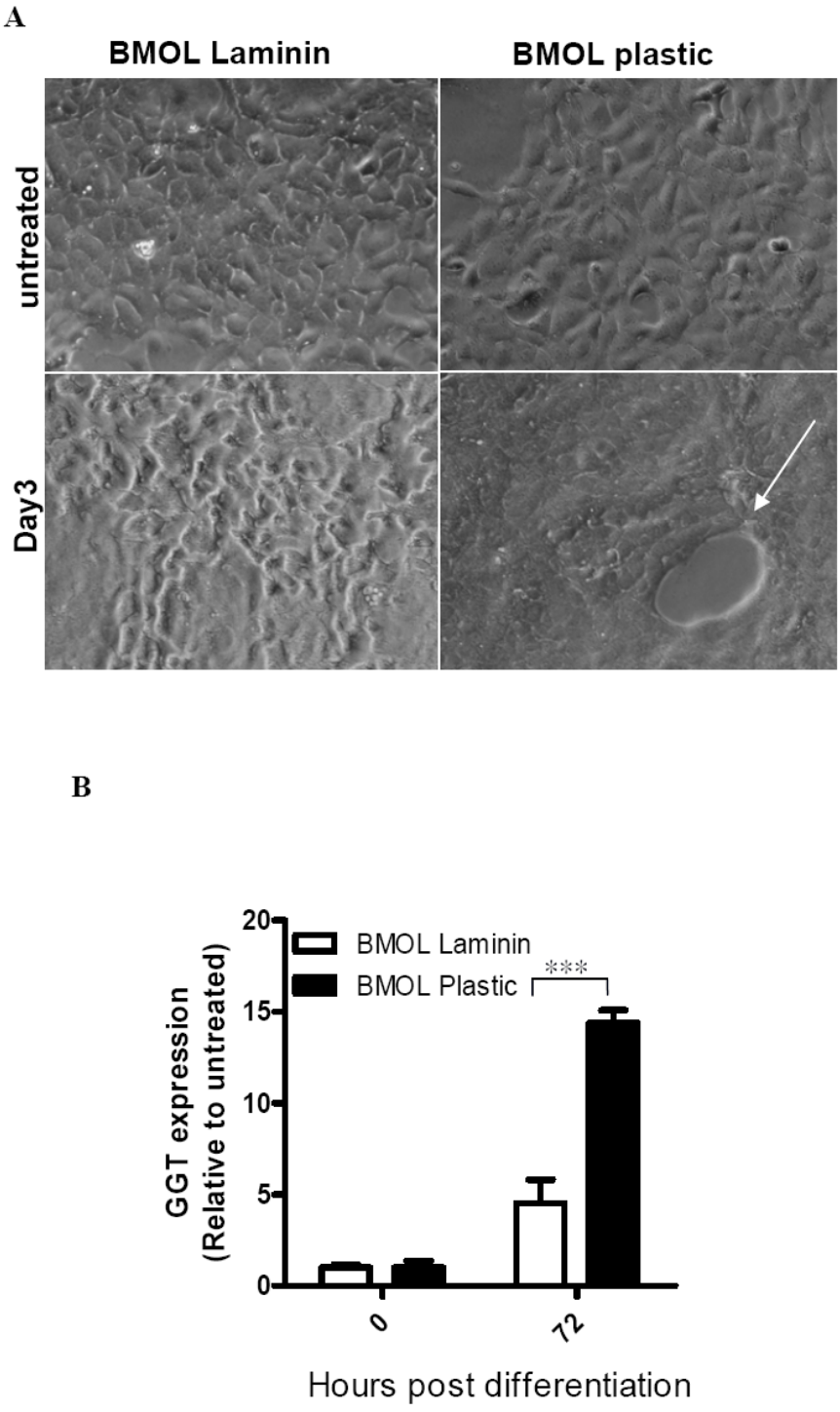

A
BMOL Laminin
BMOL plastic
untreated
Day3
B
BMOL Laminin
BMOL Plastic
***
GGT expression
(Relative to untreated)
20
15
10
5
0
0
72
Hours post differentiation

Figure 4.3

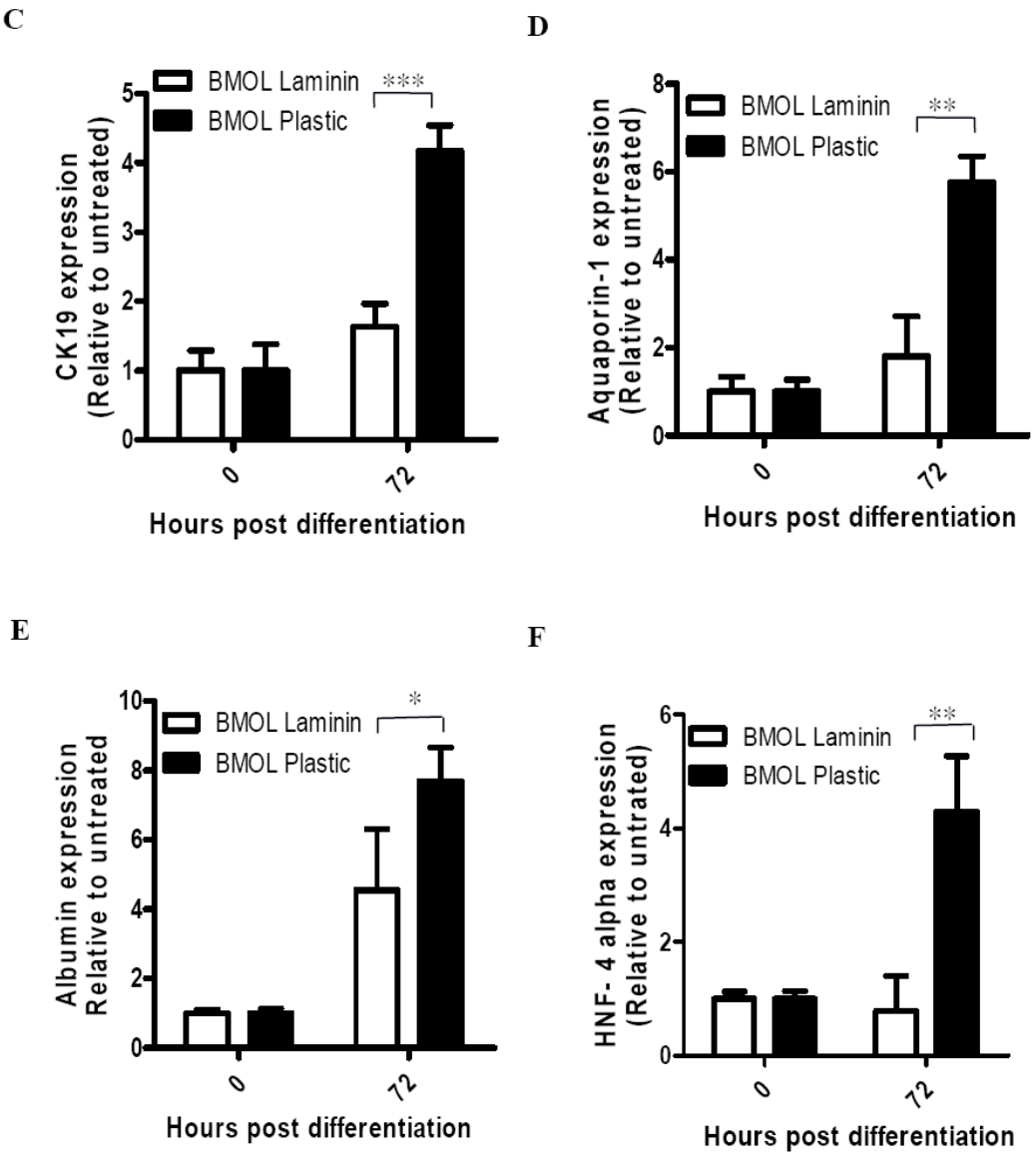

C
BMOL Laminin
BMOL Plastic
***
CK19 expression
(Relative to untreated)
0
1
2
3
4
5
0
72
Hours post differentiation
D
BMOL Laminin
BMOL Plastic
**
Aquaporin-1 expression
(Relative to untreated)
0
2
4
6
8
0
72
Hours post differentiation
E
BMOL Laminin
BMOL Plastic
*
Albumin expression
Relative to untreated
0
2
4
6
8
10
0
72
Hours post differentiation
F
BMOL Laminin
BMOL Plastic
**
HNF- 4 alpha expression
(Relative to untrated)
0
2
4
6
0
72
Hours post differentiation

Figure 4.3 BMOLs cultured on plastic plate differentiated more rapidly

(A) The differentiation treatment of 2 % matrigel, 50ng/ml EGF, and 30ng/ml OSM on BMOLs cultured on plastic or laminin-coated plates for 3 days. (B~F) Quantitative PCR analysis of the biliary cell (Aquaporin-1, GGT, and CK19) and the hepatocyte (Hnf-4α and Albumin) markers in 2 % matrigel, 50ng/ml EGF, and 30ng/ml OSM treated BMOLs cultured on plastic or laminin-coated plates for 3 days. The relative mRNA levels were compared with its basal level in the untreated BMOLs after being normalized to the housekeeping gene: PPIA. Data represent mean + SEM, n=3; Asterisk denotes a significant deviation from the mean. (Student's t-Test, *P<0.05;**P<0.01;***P<0.001)

4.3.4 Galectin-3 is required for the maintenance of BMOLs in an undifferentiated form on laminin.

To further analyse the effect of knocking down galectin-3 expression on BMOL differentiation, BMOLs cultured on laminin-coated plates were transfected with galectin-3 siRNA and scrambled siRNA as a negative control for two days then BMOLs were treated with 2 % matrigel, 50ng/ml EGF, and 30ng/ml OSM for 3 days to induce BMOL differentiation. (Fig 4.4A)

RNA was then extracted for quantitative PCR analysis. The expression of galectin-3 was analysed to ensure the knock down efficiency. More than 50 % of the galectin-3 expression was reduced by galectin-3 siRNA in undifferentiated BMOLs and BMOLs supplemented with 2% matrigel, 50ng/ml EGF, and 30ng/ml OSM. (Fig 4.4B) After 3 days of differentiation, BMOLs transfected with Galectcin-3 siRNA had higher gene expression of both the biliary cell markers: CK19, Aquaporin-1, GGT and the hepatocyte markers: Hnf-4α and albumin (Fig 4.4B-F), compared to BMOLs transefected with scrambled siRNA as a negative control. This result indicates that knock down of galectin-3 expression in BMOLs induces a more rapid differentiation into mature biliary cells and hepatocytes on laminin, suggesting galectin-3 is required for BMOLs to maintain in an undifferentiated state on laminin.

Figure 4.4

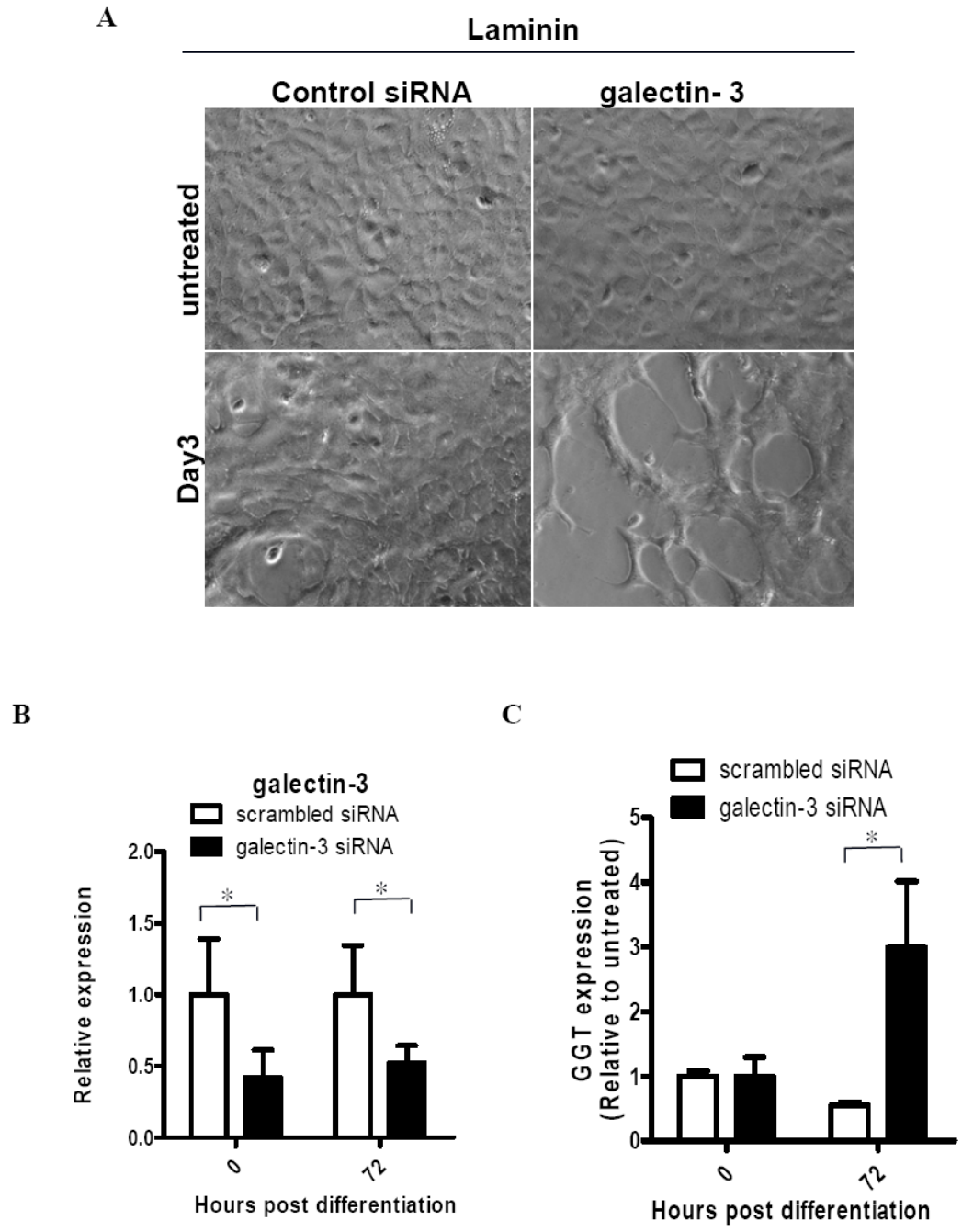

A
Laminin
Control siRNA
galectin- 3
untreated
Day3
B
galectin-3
scrambled siRNA
galectin-3 siRNA
Relative expression
2.0
1.5
1.0
0.5
0.0
*
*
0
72
Hours post differentiation
C
scrambled siRNA
galectin-3 siRNA
GGT expression
(Relative to untreated)
5
4
3
2
1
0
*
0
72
Hours post differentiation

Figure 4.4

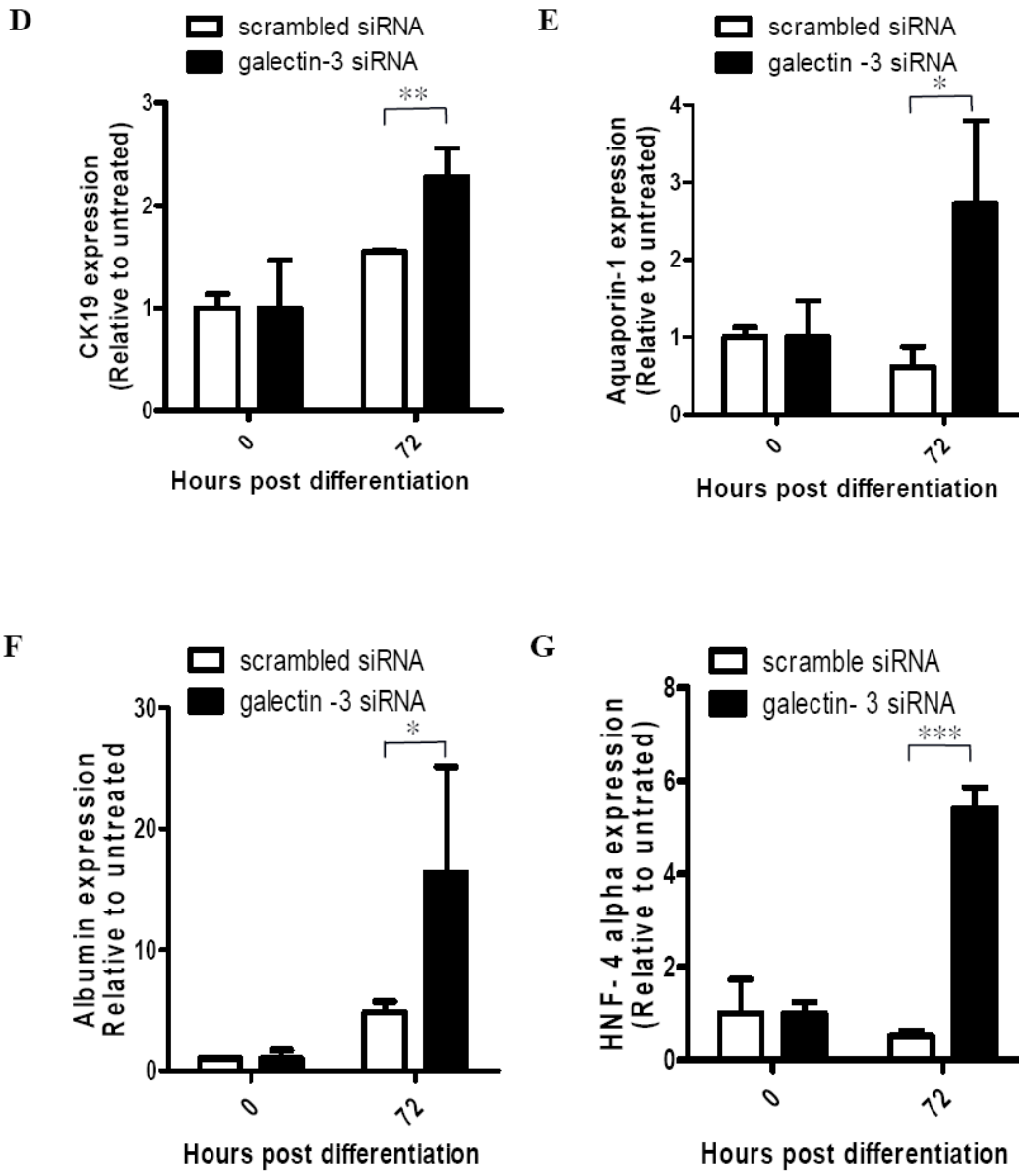

D
scrambled siRNA
galectin-3 siRNA
**
CK19 expression
(Relative to untreated)
3
2
1
0
0
72
Hours post differentiation
E
scrambled siRNA
galectin -3 siRNA
*
Aquaporin-1 expression
(Relative to untreated)
4
3
2
1
0
0
72
Hours post differentiation
F
scrambled siRNA
galectin -3 siRNA
*
Albumin expression
Relative to untreated
30
20
10
0
0
72
Hours post differentiation
G
scramble siRNA
galectin- 3 siRNA
***
HNF- 4 alpha expression
(Relative to untrated)
8
6
4
2
0
0
72
Hours post differentiation

Figure 4.4 Knock down of galectin-3 expression in BMOLs results in a more rapid differentiation

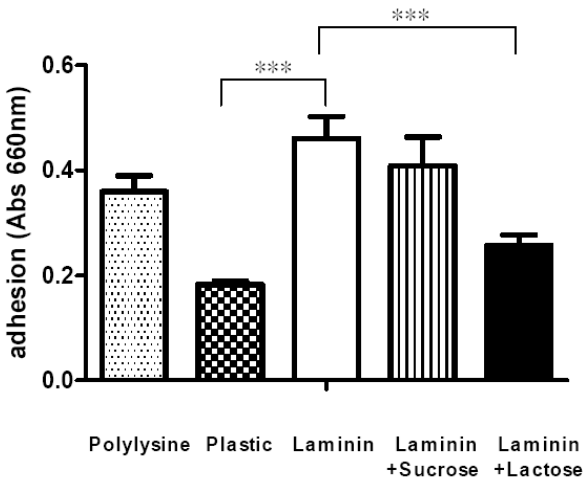
(A) The differentiation treatment of 2 % matrigel, 50ng/ml EGF, and 30ng/ml OSM on BMOLs which were transfected with scrambled and galectin-3 siRNA for 3 days on laminin-coated plates. (B) Quantitative PCR analysis of galectin-3 in undifferentiated and 2 % matrigel, 50ng/ml EGF, and 30ng/ml OSM treated BMOLs which were transfected with scrambled or galectin-3 siRNA on laminin-coated plates for 3 days. The relative mRNA levels were compared with its basal level in BMOLs which were transfected with scrambled siRNA after being normalized to the housekeeping gene: PPIA. (C~E) The biliary cell (Aquaporin-1, GGT, and CK19) and the hepatocyte (Hnf-4α and Albumin) markers in undifferentiated and 2 % matrigel, 50ng/ml EGF, and 30ng/ml OSM treated BMOLs which were transfected with scrambled or galectin-3 siRNA on laminin-coated plates for 3 days. The relative mRNA levels were compared with its basal level in undifferentiated BMOLs after being normalized to the housekeeping gene: PPIA. Data represent mean + SEM, n=3; Asterisk denotes a significant deviation from the mean (Student's t-Test, *P<0.05;**P<0.01;***P<0.001)

4.3.5 Inhibition of galectin-3 inhibits adhesion of BMOLs on laminin.

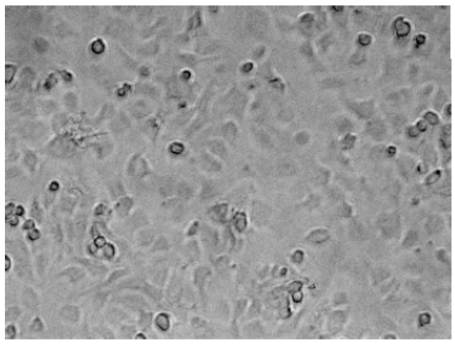
The role of the extracellular binding activity of galectin-3 in BMOL adhesion was then analysed. 96 well culture plates were coated with laminin and polylysine which serves as a positive control for complete adhesion. 1×10^5 cells /well of untreated BMOLs and BMOLs which were preincubated with 50mM lactose or 50mM sucrose for 2 hour were plated on polylysine-coated, laminin-coated, and plastic plates. After 1 hour, non-adherent cells were washed off and the adherent cells were stained by Diff-Quick stain which was designed to incorporate cytoplasmic (pink) staining with nuclear (blue) staining. The adherent cell number was then measured by absorbance at 660 nm after adding DMSO. Significantly, fewer BMOLs were adherent to plastic plates compared to laminin-coated plates. (Fig 4.5A) In addition, BMOLs adopted a flattened adherent morphology when plated on laminin-coated plates, but remained round and non well-adherent morphology when plated on plastic plates. (Fig 4.5B, C) Furthermore, preincubation of cells with lactose, a competitive inhibitor of the extracellular binding activity of galectin-3, significantly reduced the number of adherent BMOLs, compared to untreated and sucrose-treated BMOLs on laminin-coated plates.

Figure 4.5

A



B



C

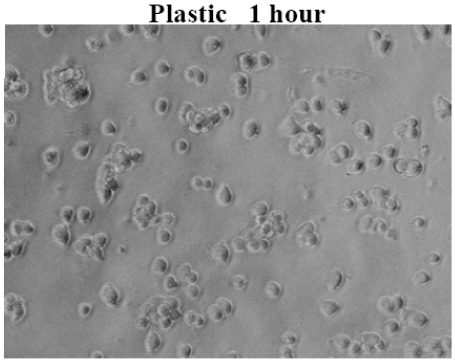


Figure 4.5 Reduction of the adherent cell numbers on laminin by treating BMOLs with lactose. (A) Adhesion of untreated and 50 mM lactose or 50 mM sucrose treated BMOLs to laminin - coated, and plastic plates. Polylysine coatings served as a positive control. Data represent mean + SEM, n=3; Asterisk denotes a significant deviation from the mean. (Student's t-Test, ***P<0.001) Morphology of BMOLs cultured on (B) laminin-coated or (C) plastic plates for 1 hour. Original magnification: x 200.

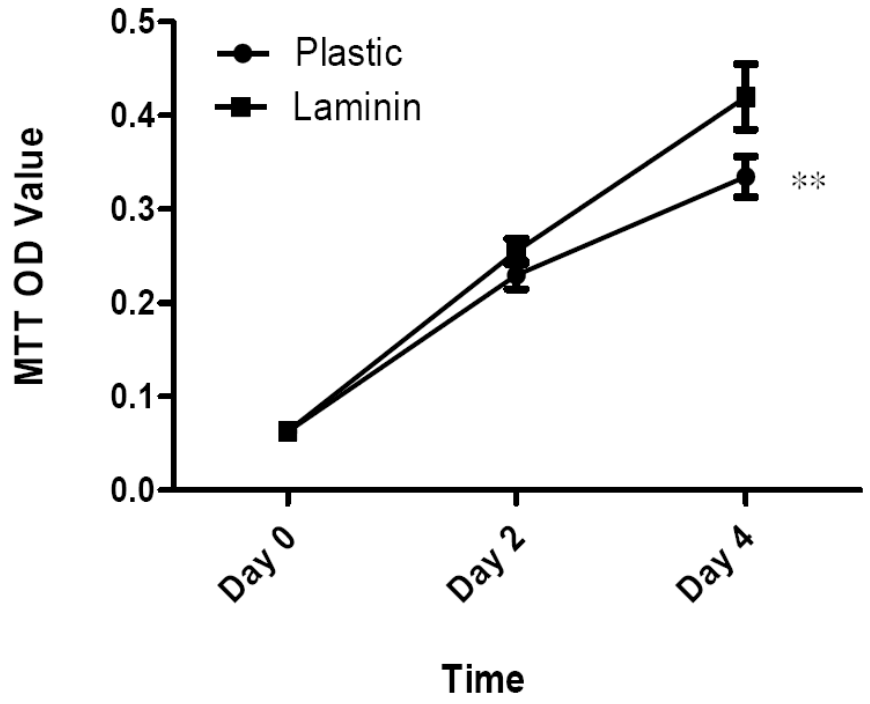
4.3.6 Both laminin and the extracellular binding actvity of galectin-3 is important for BMOL proliferation

To assess the role of laminin in BMOL proliferation, the MTT assay was used to assess BMOL proliferation. BMOLs were cultured on laminin-coated or plastic plates and the cell number was determined by a colorimetric MTT assay, as measured by absorbance at different time points: Day 0, 2 , and 4. The result indicated that MTT optical density (OD) value in the BMOLs cultured on laminin-coated plates is significantly higher than those cultured on plastic plates at day 4, suggesting the BMOLs cultured on laminin-coated plates grew faster than the BMOLs cultured on plastic plates (Fig. 4.6A) This result confirms the important role of laminin in BMOL proliferation.

I further analysed whether the extracellular binding activity of Galetin-3 is essential for BMOL proliferation on laminin-coated plates. Lactose has been shown as a competitive inhibitor of galectin-3. BMOLs which were cultured on laminin-coated plates were treated with 50mM of lactose to block the extracellular binding activity of galectin-3 or 50 mM of sucrose as a negative control. BMOL proliferation was then assessed by the MTT assay. The result showed that MTT optical density (OD) value in lactose treated BMOLs is significantly lower than the untreated BMOLs and those treated with sucrose, indicating BMOLs treated with lactose grew slower than untreated BMOLs and sucrose treated BMOLs. (Fig 4.6B)

Figure 4.6

A


0.5
0.4
0.3
0.2
0.1
0.0
MTT OD Value
Plastic
Laminin
**
Day 0
Day 2
Day 4
Time

B

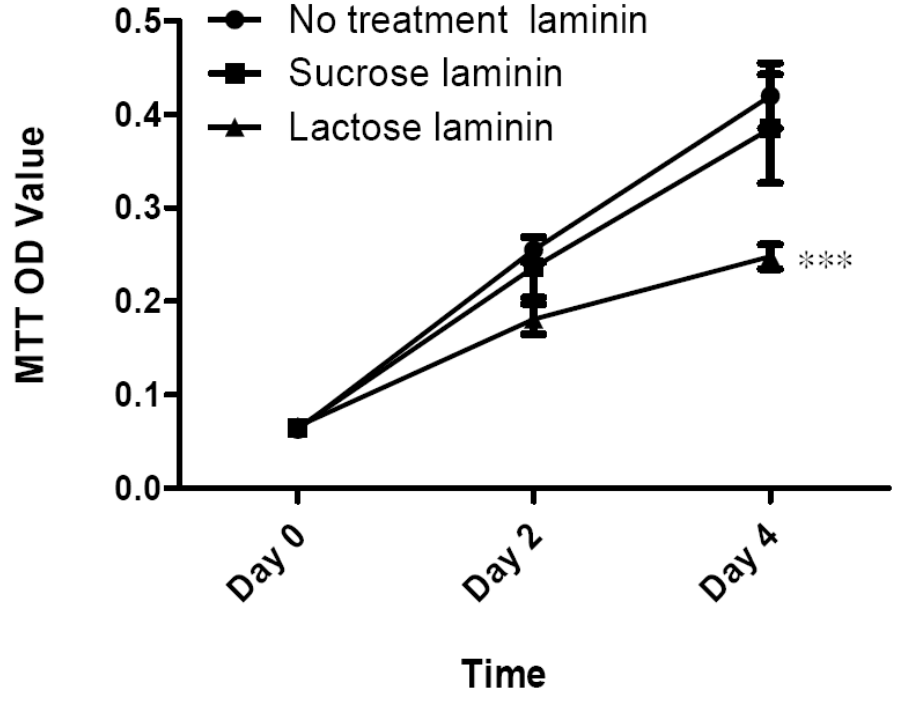

0.5
0.4
0.3
0.2
0.1
0.0
MTT OD Value
No treatment laminin
Sucrose laminin
Lactose laminin
***
Day 0
Day 2
Day 4
Time

Figure 4.6 Laminin and the extracellular binding activity of galectin-3 are both important for BMOL proliferation. (A) MTT assay in BMOLs cultured on laminin- coated and plastic plates at Day 0, 2, and 4 (B) MTT assay in BMOLs cultured on laminin- coated plates with or without treatment of 50mM lactose, 50mM sucrose (negative control) at Day 0, 2, and 4. Cells were seeded at a density of 7.5 $\times 10^3$ cells/well supplemented with growth medium containing 10% FCS. Data represent mean + SEM, n=3; Asterisk denotes a significant deviation from the mean (Student's t-Test, **P<0.01;***P<0.001)

4.4 Discussion

BMOLs were used in this chapter to further analyse the mechanisms of galectin-3 modulating LPC proliferation and differentiation since LPC line is easier to work on for studies such as knock down of galectin-3 expression by trasfecting galectin-3 siRNA or cell adhesion and proliferation assay. In fact, primary LPCs are resisted to transfection with siRNA. I firstly showed that galectin-3 was expressed by BMOLs and secreted by BMOLs extracellularly into medium. In addition, previous studies have indicated that BMOLs express both the hepatic and biliary markers, indicating their biopotentiality to differentiate into both hepatic and biliary lineages (Strick-Marchand et al., 2002). Upon the treatment of 2% matrigel, 50ng/ml EGF, and 30ng/ml OSM for 3 days, the biliary cell marker: CK19, aquaporin-1, and GGT and the hepatocyte marker: albumin and HNF4α were significantly up-regulated in BMOLs to confirm the ability of BMOLs to differentiate into both hepatic and biliary lineages.

The role of laminin in BMOL differentiation was then assessed. BMOLs differentiated into hepatic and biliary lineages more rapidly when cultured on plastic plates compared to laminin-coated plate. In addition, knock down of galectin-3 expression in BMOLs led to differentiation into both hepatic and biliary lineages more rapidly even if cultured on laminin-coated plates. This result is similar to my study of galectin-3 null primary LPC differentiation in the previous chapter, further confirming that the absence or the knock down of galectin-3 expression lead to a more rapid LPC differentiation. Moreover, I also indicated that BMOLs cultured on laminin grew faster than those cultured on plastic-coated plates which confirmed the essential role of laminin in promoting LPC proliferation. Taken together, I confirmed

that both laminin and galectin-3 are required for the maintenance of LPCs in an undifferentiated state to promote LPC proliferation. However, the exact mechanism of galectin-3 regulating LPC proliferation and differentiation remains unknown. Previous studies indicated that galectin-3 promotes neural cell adhesion on laminin and neurite growth (Penka et al., 1998). Here, I propose that galectin-3 enhance LPC adhesion to laminin to maintain LPCs in an undifferentiated state and promote LPC proliferation.

Whether galectin-3 plays an important role in BMOL adhesion to laminin was then investigated. Previous studies have shown that galectin-3 modulate cell adhesion to extracellular matrix such as laminin by interacting with various β-galactoside containing glycans via its carbohydrate recognition domain (CRD) (Hughes et al., 2001). Galectin-3-mediated effects on cell adhesion may be either positive enhancing or negative inhibitory (Hughes et al., 2001). For example, breast cancer cell with high expression of galectin-3 interact well and spread very rapidly on ECM proteins compared to those with low or no galectin-3 expression (Warfield et al., 1997; Matarrese et al., 2000; Honjo et al., 2001). Galectin-3 significantly increased the adhesion of human neutophils to various substrata (Lotan et al., 1994; Kuwabara et al., 1996). However, galectin-3 has been shown to reduce the adhesion of myoblasts and kidney epithelial cells to laminin (Sato et al., 1992).

Here, I have found that laminin profoundly promoted BMOL adhesion. In addition, treating BMOLs with lactose significantly reduced BMOL adhesion to laminin, suggesting that the extracellular binding activity of galectin-3 has a positive effect on BMOL adhesion to laminin. Moreover, the role of the extracellular binding activity

of Galetin-3 in BMOL proliferation was also assessed. Compared to the untreated and sucrose- treated BMOLs, the lactose-treated BMOLs grew slower after 4 days of culture, suggesting the extracellular binding activity of Galetin-3 is essential for BMOL proliferation. Taken together, these data suggest the extracellular binding activity of galectin-3 is important for LPC adhesion to laminin and LPC proliferation.

However, it is still not clear how galectin-3 regulates BMOL adhesion to laminin. Galectin-3 can modulate cell adhesion by binding to the molecules involved in cell adhesion directly or by steric hindrance of the interaction between molecules involved in cell-cell or cell-matrix adhesion. The mechanism may also involve integrin activation (Hughes et al., 2001, detail mechanism is shown in the chapter one). Whether galectin-3 modulation of BMOL adhesion to laminin involves integrin activation remains unknown. Further research needs to be conducted to answer this question.

In summary, I confirmed the regulating role of galectin-3 in LPC proliferation and differentiation based on the finding in this chapter and the previous chapter, either by investigating galectin-3 null primary LPCs or knocking down galectin-3 expression in BMOLs. Galectin-3 is important for LPC proliferation, but the absence of galectin-3 leads LPCs to differentiate into both lineages more rapidly on laminin. These data not only confirm that galectin-3 is required to maintain LPCs in an undifferentiated state but also suggest that galectin-3 is crucial in mediating the homeostatic balance between proliferation and differentiation of LPCs. In addition, I also showed that competitive inhibition of galectin-3 inhibited adhesion and

proliferation of LPCs on laminin, suggesting that galectin-3 is required for LPCs to attach laminin and promote proliferation.

CHAPTER 5

INVESTIGATION THE ROLE OF GALETIN-3 IN REGULATING CELL CYCLE AND CELL-ADHESION SIGNALLING PATHWAY

5.1 Abstract

The importance of galectin-3 in maintaining LPC in an undifferentiated state on laminin has been demonstrated in the first and second chapter. Additionally, the second chapter demonstrated the role played by the extracellular binding activity of galectin-3 in promoting LPC proliferation and enhancing LPC adhesion to laminin. However, the mechanism of galectin-3 regulating LPC proliferation was not completely investigated. Galectin-3 may regulate LPC proliferation by regulating the activity of cell cycle regulators or cell-adhesion mediated signalling pathways. In addition, galectin-3 may also indirectly mediate β1 integrin activation by binding to and activating the heterodimeric transmembrane amino acid transporter CD98. This chapter demonstrates that the absence of galectin-3 or the knock down of galectin-3 expression leads to down-regulation of cyclin D1 and up-regulation of p16 and p21. In addition, down-regulation of FAK and Akt phosphorylation was observed in galectin-3 null LPCs and galectin-3 siRNA transfected BMOLs. Moreover, CD98 and integrin β1 expression are strongly associated with activation of LPCs. Also, CD98 and integrin β1 are expressed by panCK and galectin-3 positive cells *in vitro*, especially on dividing cells. These results suggest that integrin β1 and CD98 may be involved in the regulation of LPC proliferation.

5.2 Introduction

Galectin-3 is expressed ubiquitously in the cytoplasm, the nucleus, extracellular compartment, and at the cell surface. Thus, galectin-3 medicates multiple functions depending on its subcellular localization. Generally, extracellular galectin-3 modulates cell adhesion and cell - cell interactions via its CRD domain, while intracellular galectin-3 mediates several signal transduction pathway (Ochieng et al., 2004; Nakahara et al., 2005; Susumu et al., 2007). Nuclear galectin-3 has been found as a nuclear matrix protein and involved in pre-mRNA splicing (Park et al., 2001; Wang et al., 2004; Paces-Fessy et al., 2004; Susumu et al., 2007). The mechanism in which galectin-3 regulates LPC proliferation is not completely known. However, the previous chapter demonstrated that the extracellular binding activity of galectin-3 is important for LPC proliferation and LPC adhesion to laminin. Galectin-3 may regulate LPC proliferation by mediating LPC adhesion to laminin. It is also possible that extracellular galectin-3 cross-links surface glygoproteins such as signaling molecules kinases and transduces signals to regulate LPC proliferation. Furthermore, the nuclear galectin-3 may also regulate LPC proliferation intracellularly. Galectin-3 has been shown to shuttle between the nucleus and cytoplasm and is engaged in pre-mRNA splicing and the regulation of cancer-related gene expression (Susumu et al., 2007; Lin et al., 2002; Paron et al., 2003; Song et al., 2003) or cell growth, apoptosis, and cell-cycle progression (Dagher et al., 1995; Wang et al., 2004). Galectin-3 might modulate transcription factors to regulate cyclin D or c-myc gene expression which are involved in cell proliferation.

Galectin-3 has been reported to bind integrins and regulates β1 mediated adhesion to ECM (Friedrichs et al, 2008; Saravanan et al., 2009). In addition, galectin-3 may also

indirectly mediate integrin β1 activation by binding to the heterodimeric transmembrane amino acid transporter CD98 (Dong et al, 1997; MacKinnon et al., 2008). CD98, a ligand for galectin-3 and an endogenous target for galectin-3 in activaton of integrins, may be involved in the regulation of LPC proliferation. Galectin-3 may mediate the binding activity of CD98 with integrin β1 and activate integrin-mediated pathway to modulate LPC adhesion to laminin and enable LPC proliferation.

5.3 Results

5.3.1. Galectin-3 regulates the expression of cyclin D1, p16 and p21

In order to further investigate the role of galectin-3 in LPC proliferation, the expression of cell cycle regulators were examined. Both WT and galectin-3 null LPCs were isolated from the livers of the CDE-fed WT and galectin-3 null mice. After seven days of culture when primary LPCs adopted oval morphology with a high nuclei/cytoplasm ratio, both WT and galectin-3 null primary LPCs were cultured on laminin-coated or plastic plates for another 4 days. Total cell lysates were then collected from WT and galectin-3 null LPCs. Immunoblot analysis showed that cyclin D1 protein expression was down-regulated in galectin-3 null LPCs either cultured on laminin-coated or plastic plates. (Fig 5.1A, B) In addition, the gene expression of cell cycle inhibitors p21 and p16 were also examined. RNA from WT and galectin-3 null primary LPCs which were cultured with serum free medium for 1 day (time zero) or medium contains 5% serum for 4 days, respetively, was extracted for quantitative PCR analysis. The expression of p21 and p16 were both up-regulated in galectin-3 null LPCs which were either cultured on laminin-coated or plastic plates for 4 days. (Fig 5.1B-E)

Figure 5.1

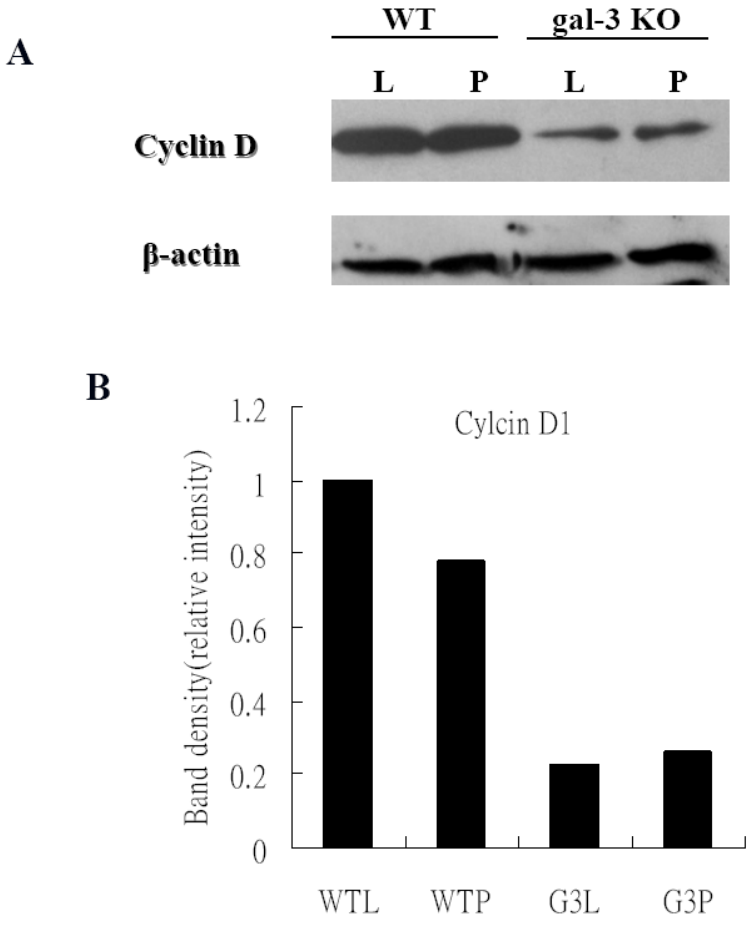

A
WT
gal-3 KO
L
P
L
P
Cyclin D
β-actin
B
Cylcin D1
Band density(relative intensity)
1.2
1
0.8
0.6
0.4
0.2
0
WTL
WTP
G3L
G3P

Figure 5.1

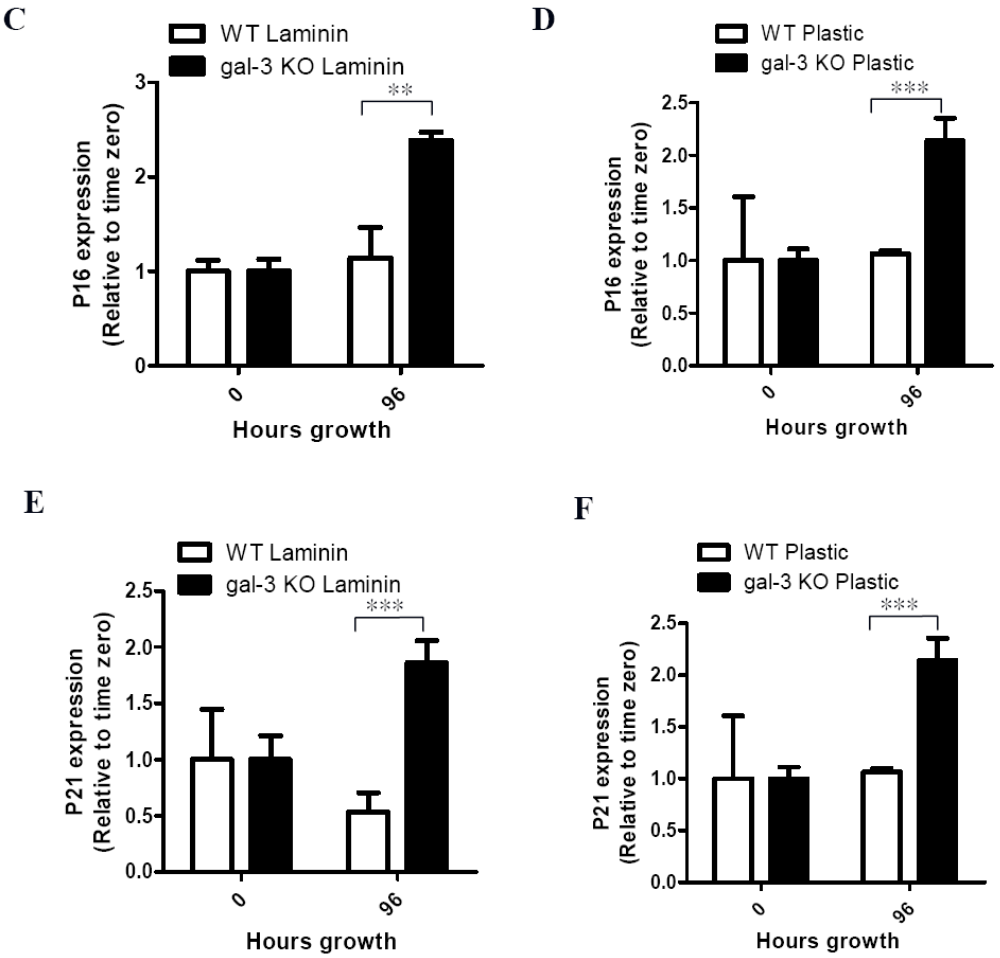

C
WT Laminin
gal-3 KO Laminin
**
P16 expression
(Relative to time zero)
3
2
1
0
0
96
Hours growth
D
WT Plastic
gal-3 KO Plastic
***
P16 expression
(Relative to time zero)
2.5
2.0
1.5
1.0
0.5
0.0
0
96
Hours growth
E
WT Laminin
gal-3 KO Laminin
***
P21 expression
(Relative to time zero)
2.5
2.0
1.5
1.0
0.5
0.0
0
96
Hours growth
F
WT Plastic
gal-3 KO Plastic
***
P21 expression
(Relative to time zero)
2.5
2.0
1.5
1.0
0.5
0.0
0
96
Hours growth

Figure 5.1 Galectin-3 down-regulated cyclin D1 but up-regulated p16 and p21.

(A) Lysates (10μg/lane) of WT and galectin-3 null primary LPCs which were cultured on laminin-coated plates (L) or plastic plates (P) for 4 days were subjected to immunoblot analysis with an anti-cyclin D1, and anti-β-actin antibodies. (B) Respective densitometry. Quantitative PCR analysis of P16 in WT and galectin-3 null LPCs which were cultured on (C) laminin-coated plates or (D) plastic plates for 4 days. Quantitative PCR analysis of P21 in WT and galectin-3 null LPCs which were cultured on (E) laminin-coated or (F) plastic plates for 4 days. The relative mRNA levels were compared with WT and galectin-3 null LPCs which were cultured in serum-free cultured medium after being normalized to the housekeeping gene: PPIA. Data represent mean + SEM, n=3; Asterisk denotes a significant deviation from the mean (Student's t-Test, ** $p<0.01$; *** $p<0.001$)

5.3.2 A decrease in phosphorylation of FAK, Akt was observed in galectin-3 null LPCs when cultured on plastic plates.

Cell adhesion-mediated cyclin D_1 promoter activation appears to occur through focal adhesion kinase (Zhao *et al*., 1998). The tyrosine phosphorylation of the integrin-linked FAK and its downstream kinases Akt has been shown to play an important role in adhesion-mediated signal transduction (Schwartz et al., 2002). This study addressed the question as to which cell adhesion-mediated signalling pathwaywas regulated by galectin-3.

As shown in figure 5.2, the phosphorylation of FAK was reduced in galectin-3 null LPCs when cultured on plastic plates, but only a slight reduction can be observed in galectin-3 null LPC when cultured on laminin-coated plates (Fig 5.2 A, B). Similarly, an obvious decrease in Akt phosphorylation was observed in the galectin-3 null LPCs when cultured on plastic plates but there was only a slight reduction of Akt phosphorylation in the galectin-3 null LPCs when cultured on laminin-coated plates. (Fig 5.2 A, C)

Figure 5.2

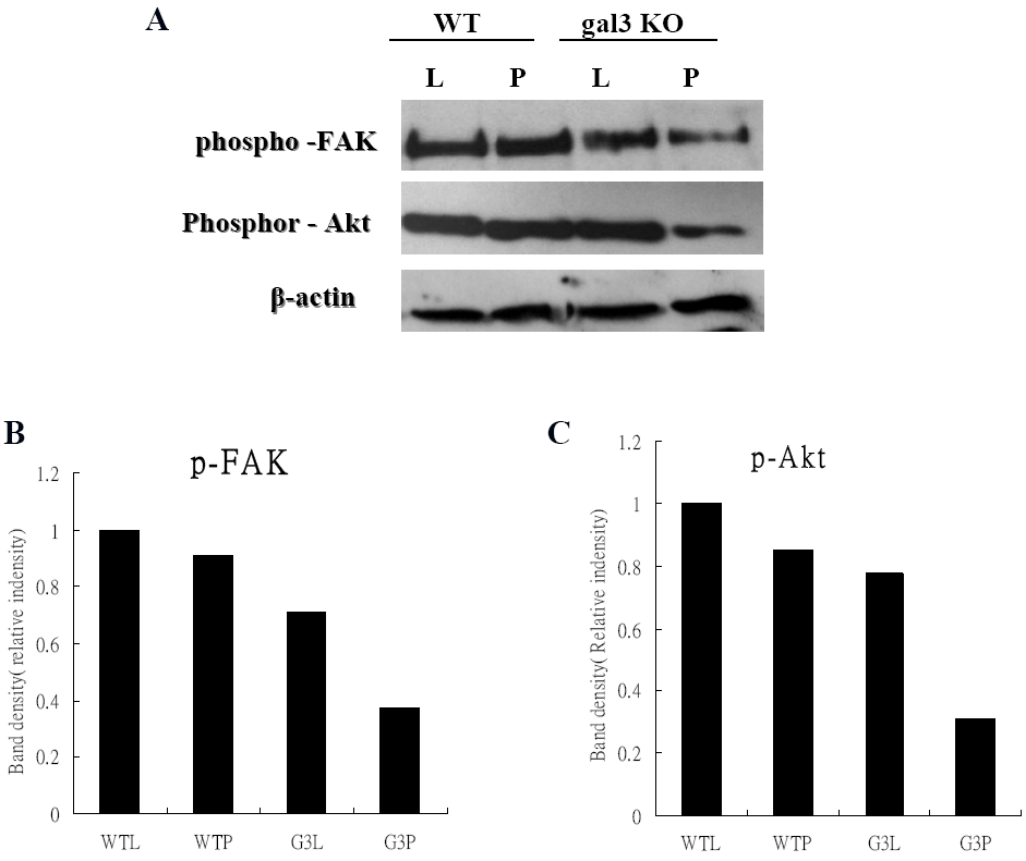

A
WT
gal3 KO
L
P
L
P
phospho -FAK
Phosphor - Akt
β-actin
B
p-FAK
Band density(relative indensity)
1.2
1
0.8
0.6
0.4
0.2
0
WTL
WTP
G3L
G3P
C
p-Akt
Band density(Relative indensity)
1.2
1
0.8
0.6
0.4
0.2
0
WTL
WTP
G3L
G3P

Figure 5.2 Western blots showing levels of the expression of phosphorylation of FAK and Akt in WT and galectin-3 null LPCs when cultured on laminin-coated or plastic plates (A) Lysates (10μg/lane) of WT and galectin-3 null primary LPCs which were cultured on laminin-coated or plastic plates for 4 days were subjected to immunoblot analysis with an anti-phospho FAK, anti-phospho Akt, and anti- β-actin antibodies. (B, C) Respective densitometry

5.3.3 No difference in activation of β-catenin and G3K3β was observed in galectin-3 null LPCs

It has been shown that galectin-3 plays a role in Wnt signaling in breast cancer by interacting with β-catenin. Galectin-3 binds to β-catenin and colocalize it in the nucleus to stimulate the expression of cyclin D1 (Shimura *et al.,* 2004). Thus the role of galectin-3 in β-catenin and GSK3β activation during LPC proliferation was investigated. In order to achieve this, the expression of active β-catenin and phosphorylation of GSK3β was assessed. Immunoblot analysis of total cell lysates showed that both the expression of active β-catenin and phosphorylation of GSK3 were comparable between WT and galectin-3 null primary LPCs cultured either on laminin-coated or plastic plates. (Fig 5.3 A-C)

Figure 5.3

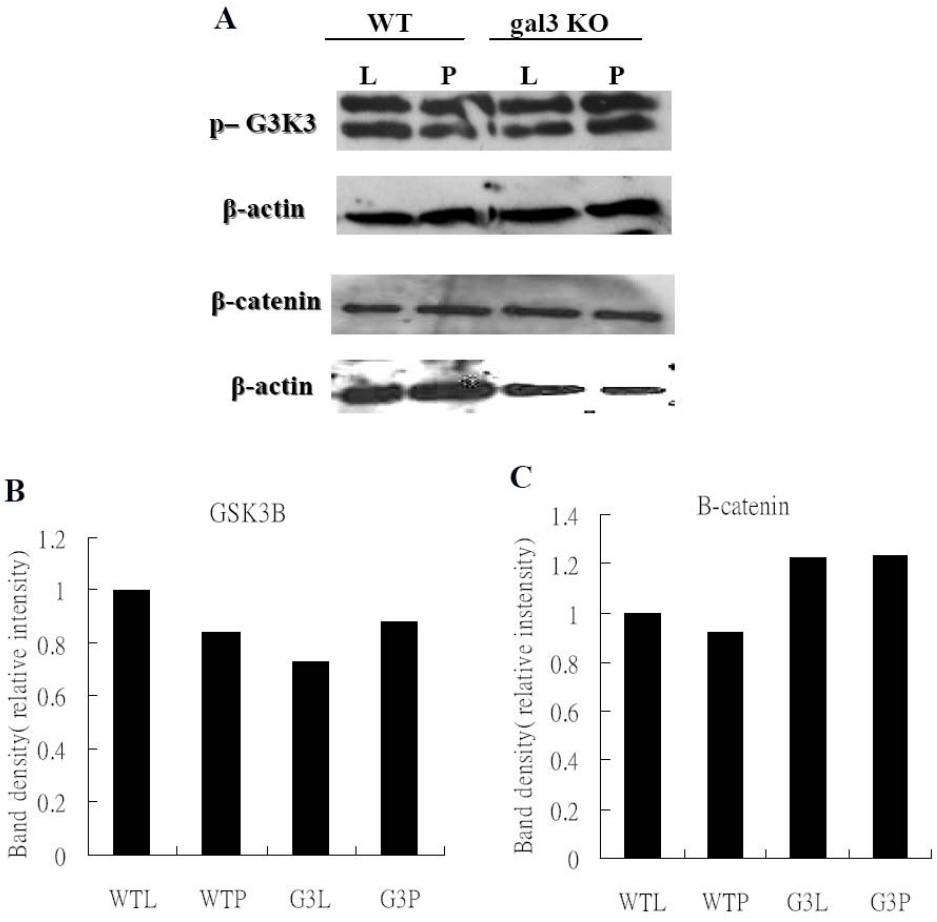

A
WT
gal3 KO
L
P
L
P
p– G3K3
β-actin
β-catenin
β-actin
B
GSK3B
Band density(relative intensity)
1.2
1
0.8
0.6
0.4
0.2
0
WTL
WTP
G3L
G3P
C
B-catenin
Band density(relative instensity)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
WTL
WTP
G3L
G3P

Figure 5.3 Western blots showing no difference in the expression of active β-catenin and phosphorylation of GSK3 in WT and galectin-3 null LPCs when cultured on laminin-coated or plastic plates (A) Lysates (10μg/lane) of WT and galectin-3 null primary LPCs which were cultured on laminin-coated or plastic plates for 4 days were subjected to immunoblot analysis with an anti- active β-catenin, anti-phospho GSK3β and anti- β-actin antibodies. (B, C) Respective densitometry

5.3.4 Knock down of galectin-3 expression down-regulated cyclin D1 and up-regulated p16 and p21.

In order to further confirm the role of galectin-3 in regulating the expression of cyclin D1, p16, and p21, galectin-3 expression was knocked down in LPC line: BMOLs by transfecting galectin-3 siRNA into BMOLs. BMOLs which were cultured on laminin-coated or plastic plates were transfected with scrambled siRNA or galectin-3 siRNA. Total RNA and cell lysates were collected after 1, 3, and 5 days of transfection.

To confirm the galectin-3 knocked down efficiency, galectin-3 protein expression was analyzed in BMOLs after transfection. (Fig 5.4A-B, D-E) The efficiency of knocking down galectin-3 expression was further confirmed by quantitative PCR analysis, demonstrating that the mRNA level of galectin-3 was significantly reduced after transfection. (Fig 5.4 G, H) The cyclin D1 protein expression was then further analyzed in BMOLs after transfection. Cyclin D1 expression was reduced in BMOLs, either cultured on laminin-coated or plastic plates after knocking down galectin-3 expression for 5 days. (Fig 5.4A, C, D, F)

The mRNA levels of p16 and p21 in the transfectants were also analyzed. P16 expression was significantly up-regulated in BMOLs which were transfected with galectin-3 siRNA and cultured either on laminin-coated (Fig 5.4 I) or plastic plates (Fig 5.4 J) after 3 and 5 days of transfection. P21 expression was also significantly up-regulated after 3 days of transfection. (Fig 5.4 L, K) Interestingly, the mRNA level of p21 was reduced in both BMOLs transfected with scrambled siRNA or galectin-3 siRNA after 5 days of transfection either on laminin-coated plates (Fig 5.4

L) or plastic plates (Fig 5.4 K) However, the mRNA level of p21 in BMOLs transfected with galectin-3 siRNA was still significantly higher than BMOLs transfected with scrambled siRNA. (Fig 5.4 L, K)

Figure 5.4

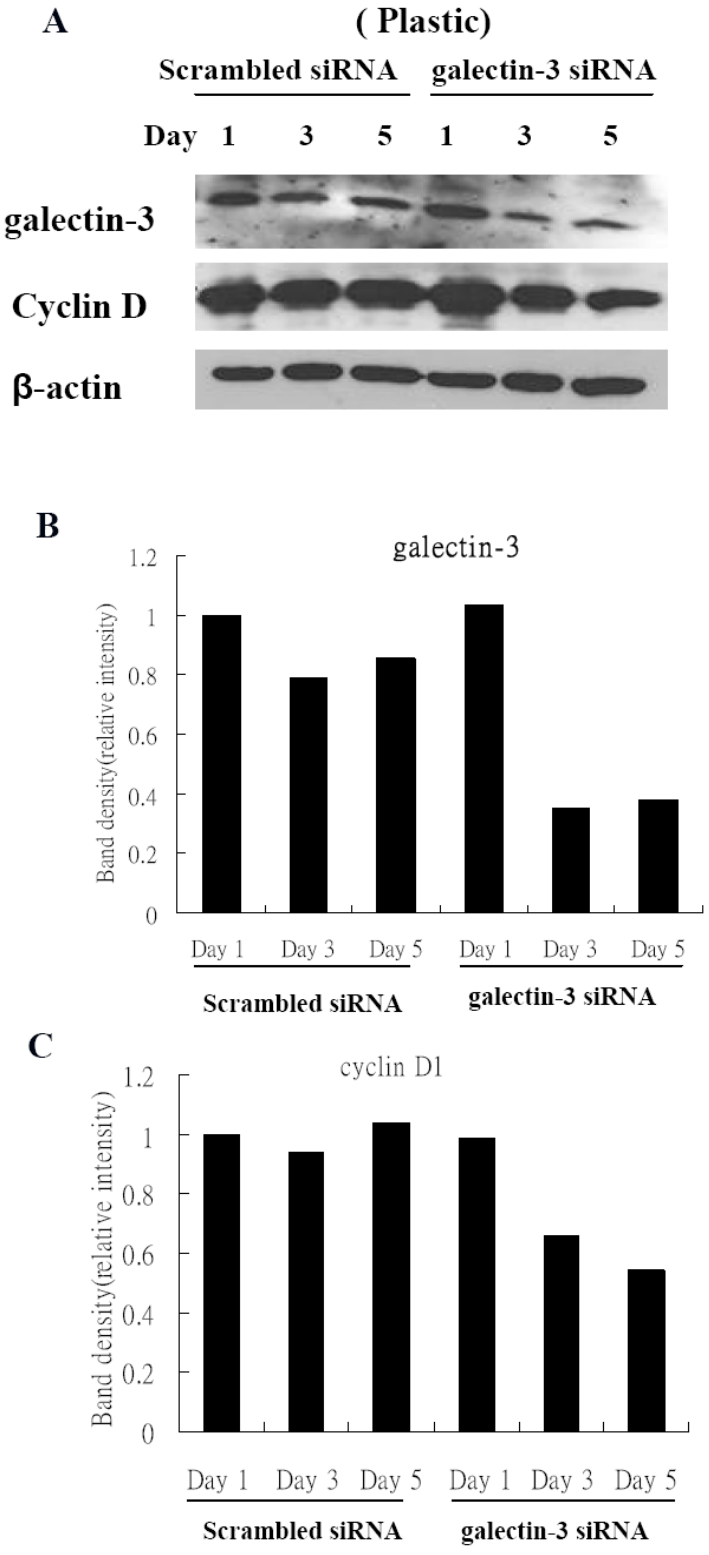

A
(Plastic)
Scrambled siRNA
galectin-3 siRNA
Day 1 3 5 1 3 5
galectin-3
Cyclin D
β-actin
B
galectin-3
Band density(relative intensity)
1.2
1
0.8
0.6
0.4
0.2
0
Day 1 Day 3 Day 5 Day 1 Day 3 Day 5
Scrambled siRNA
galectin-3 siRNA
C
cyclin D1
Band density(relative intensity)
1.2
1
0.8
0.6
0.4
0.2
0
Day 1 Day 3 Day 5 Day 1 Day 3 Day 5
Scrambled siRNA
galectin-3 siRNA

Figure 5.4

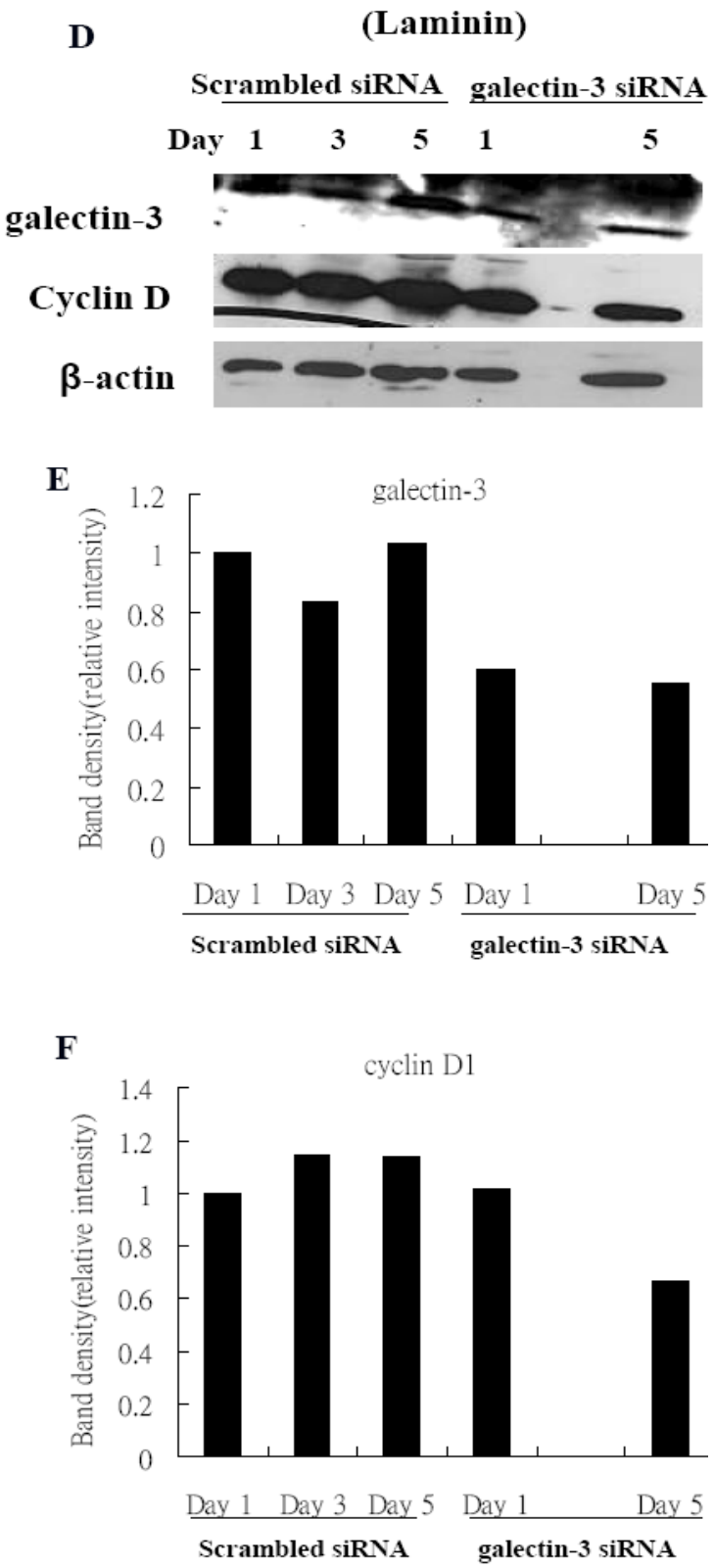
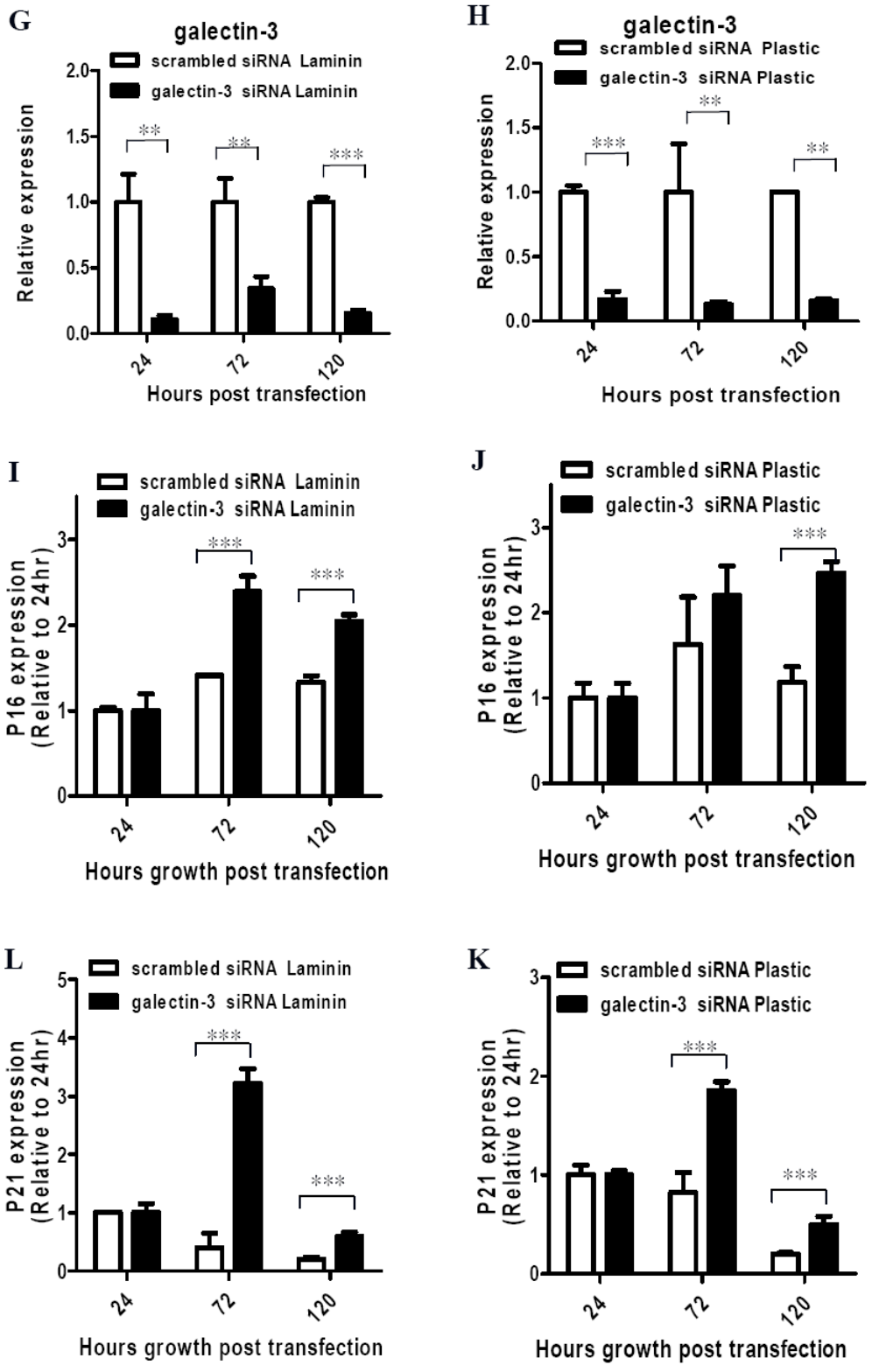

D
(Laminin)
Scrambled siRNA
galectin-3 siRNA
Day 1 3 5 1 5
galectin-3
Cyclin D
β-actin
E
galectin-3
Band density(relative intensity)
1.2
1
0.8
0.6
0.4
0.2
0
Day 1 Day 3 Day 5 Day 1 Day 5
Scrambled siRNA
galectin-3 siRNA
F
cyclin D1
Band density(relative intensity)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day 1 Day 3 Day 5 Day 1 Day 5
Scrambled siRNA
galectin-3 siRNA

Figure 5.4



165

Figure 5.4 Knock down of galectin-3 expression in BMOLs down-regulated cyclin D1 but up-regulated p16 and p21.

Lysates (10μg/lane) of BMOLs which were cultured on (A) plastic or (D) laminin-coated plates and trasfected with scrambled or galectin-3 siRNA for 1, 3, and 5 days were subjected to immunoblot analysis with an anti- galectin-3, anti-cyclin D1, and anti- β-actin antibodies. (B-C, E-F) Respective densitometry Quantitative PCR analysis of galectin-3 in BMOLs which were cultured on (G) laminin-coated or (H) plastic plates after 1, 3, and 5 days of transfection. Quantitative PCR analysis of p16 in BMOLs which were cultured on (I) laminin-coated or (J) plastic plates after 1, 3, and 5 days of transfection. Quantitative PCR analysis of p21 in BMOLs which were cultured on (L) laminin-coated or (K) plastic plates after 1, 3, and 5 days of transfection. The relative mRNA levels were compared with transfectants which were transfected with scrambled siRNA or galectin-3 siRNA for 1 day after being normalized to the housekeeping gene: PPIA. Data represent mean + SEM, n=3; Asterisk denotes a significant deviation from the mean (Student's t-Test, * $p<0.05$; ** $p<0.01$; *** $p<0.001$)

5.3.5 A decrease in FAK and Akt phosphorylation was observed in galectin-3 siRNA-transfected BMOLs which were cultured on plastic plates.

The phosphorylation of FAK and Akt was also investigated after transfecting galectin-3 siRNA into BMOLs which were cultured on laminin-coated or plastic plates for 1, 3, and 5 days. Phosphorylation of Akt was significantly reduced in BMOLs cultured on plastic plates after 3 and 5 days of transfection. (Fig 5.5A, B), while was only reduced slightly in BMOLs cultured on laminin-coated plates after transfection. (Fig 5.5 D, E)

Interestingly, FAK phosphorylation was increased in BMOLs which were transfected with scrambled siRNA, but reduced in BMOLs which were transfected with galectin-3 siRNA when cultured on plastic plates. (Fig 5.5A, C) However, there is only a slight reduction in phosphorylation of FAK after knocking down galectin-3 expression in BMOLs which were cultured on laminin-coated plates. (Fig 5.5 D, F)

Figure 5.5

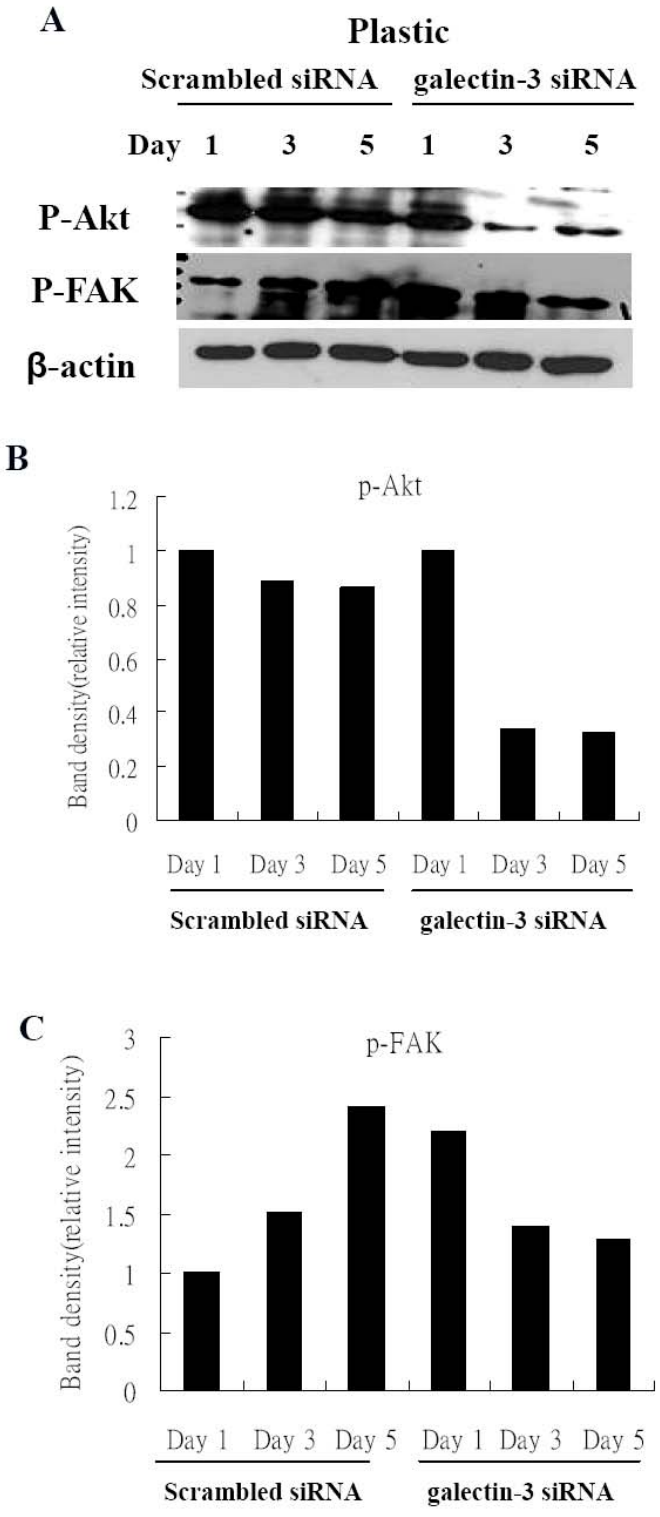

A
Plastic
Scrambled siRNA
galectin-3 siRNA
Day 1 3 5 1 3 5
P-Akt
P-FAK
β-actin
B
p-Akt
Band density(relative intensity)
1.2
1
0.8
0.6
0.4
0.2
0
Day 1 Day 3 Day 5 Day 1 Day 3 Day 5
Scrambled siRNA
galectin-3 siRNA
C
p-FAK
Band density(relative intensity)
3
2.5
2
1.5
1
0.5
0
Day 1 Day 3 Day 5 Day 1 Day 3 Day 5
Scrambled siRNA
galectin-3 siRNA

Figure 5.5

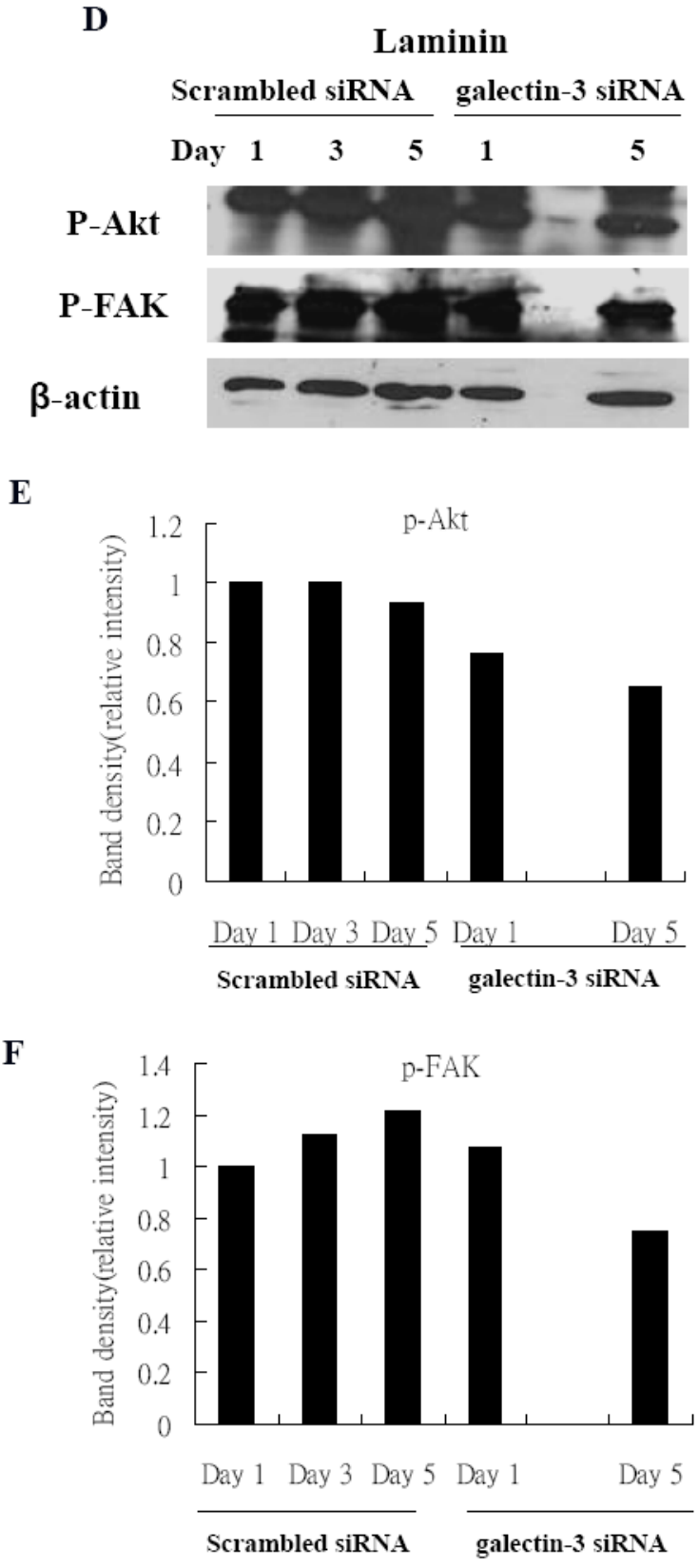

D
Laminin
Scrambled siRNA
galectin-3 siRNA
Day 1 3 5 1 5
P-Akt
P-FAK
β-actin
E
p-Akt
Band density(relative intensity)
1.2
1
0.8
0.6
0.4
0.2
0
Day 1 Day 3 Day 5 Day 1 Day 5
Scrambled siRNA
galectin-3 siRNA
F
p-FAK
Band density(relative intensity)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Day 1 Day 3 Day 5 Day 1 Day 5
Scrambled siRNA
galectin-3 siRNA

Figure 5.5 A decrease in phosphorylation of FAK and Akt was observed in galectin-3 siRNA transfected BMOLs cultured on plastic plates

Lysates (10μg/lane) of BMOLs which were cultured on (A) plastic or (D) laminin-coated plates and trasfected with scrambled siRNA or galectin-3 siRNA for 1, 3, and 5 days were subjected to immunoblot analysis with an anti-phospho FAK, anti-Akt and anti-β-actin antibodies. (B-C, E-F) Respective densitometry

5.3.6 CD98 and integrin β1 may be involved in the regulation of LPC behavior.

It has been proposed that galectin-3 is a ligand for the glycosylated extracellular domain of CD98hc and is a natural activator of CD98 (Dalton et al., 2007). Thus, galectin-3 may synergize with CD98 in the activation of integrins. The expression of CD98 in the liver of CDE-fed mice was examined by immunochemistry. CD98 was expressed around the panCK- positive LPCs. (Fig 5.6 A, B) Double immunostaining for PanCK and CD98 further confirmed that CD98 was expressed around panCK-positive LPCs. (Fig 5.6 C) In order to identify the cell type that express CD98, the immunostaining for Desmin and Von Willebrand Factor (VWF) was conducted to identify the hepatic stellate cells and endothelial cells, respectively, on serial sections of livers from CDE-fed WT mice. CD98 was expressed around the hepatic stellate cells (Fig 5.6 D, E and 5.6 G, F); however, it's not very clear whether CD98 was expressed around the VWF-positive endothelial cells. (Fig 5.6 E, F, C and 5.6 G, I)

Moreover, the expression of CD98 was increased and especially localized around the LPC reaction in the liver of CDE-fed WT mice, compared to the liver of normal WT mice. (Fig 5.7A) However, the expression pattern of CD98 in the liver of CDE-fed galectin-3 mice is similar to the liver of normal galectin-3 null mice. No obvious increase in the expression of CD98 in the liver of CDE-fed galectin-3 null mice can be observed. (Fig 5.7A)

Furthermore, the expression of integrin β1 in the liver of CDE-fed WT mice was investigated. It was observed that integrin β1 was highly expressed on hepatocytes but not on bile ducts in the liver of normal WT mice, whereas its expression was specifically restricted to the bile ducts in the liver of CDE-fed WT mice. (Fig 5.7B)

However, integrin β1 was widely expressed on hepatocytes in the liver of both normal and CDE-fed galectin-3 null mice. (Fig 5.7B)

Figure 5.6

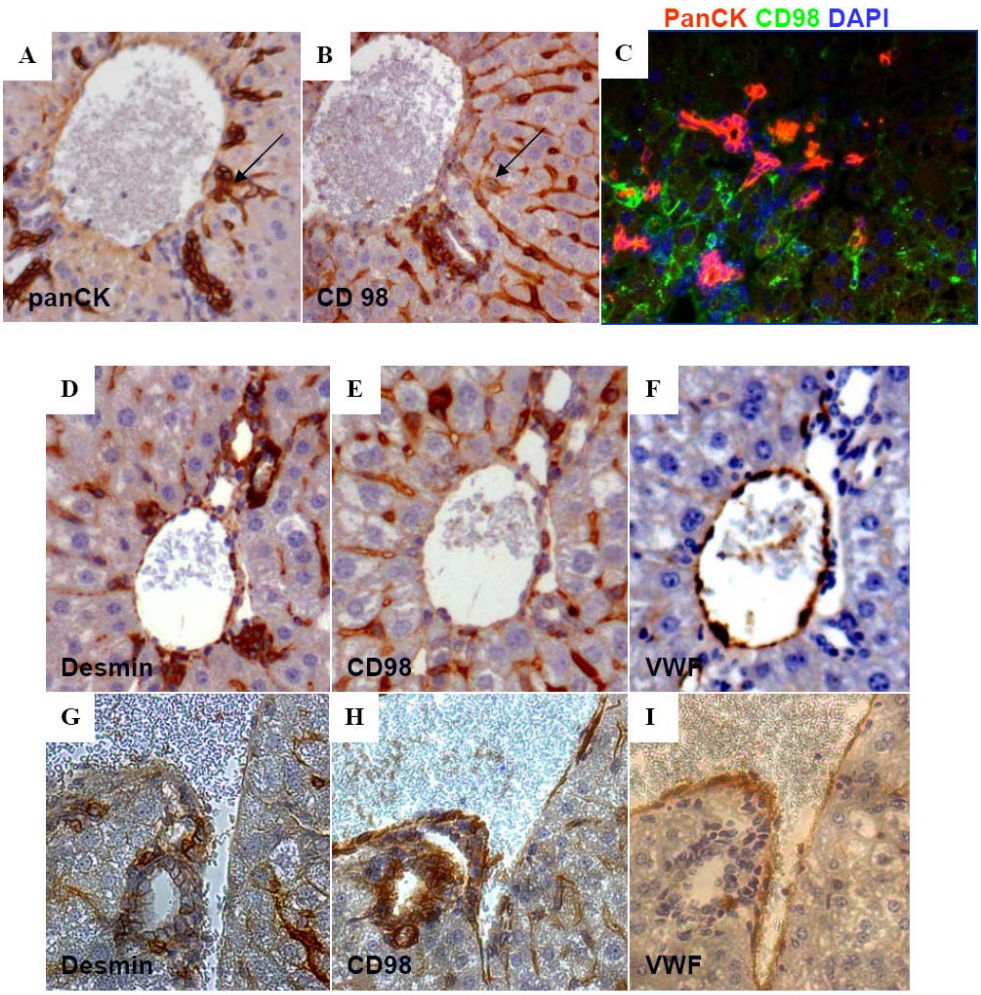

A
B
C
PanCK CD98 DAPI
panCK
CD 98
D
E
F
Desmin
CD98
VWF
G
H
I
Desmin
CD98
VWF

Figure 5.6 CD98 was expressed around LPCs and hepatic stellate cells. Immunostaining for (A) PanCK (B) CD98 on serial sections of livers from CDE-fed WT mice (C) Double immunostaining for PanCK and CD98 in the liver of CDE-fed WT mice. Immunostaining for (D, G) Desmin (E, H) CD98 (F, Q) VWF on serial sections of livers from CDE-fed mice. Original magnification: x 200.

Figure 5.7

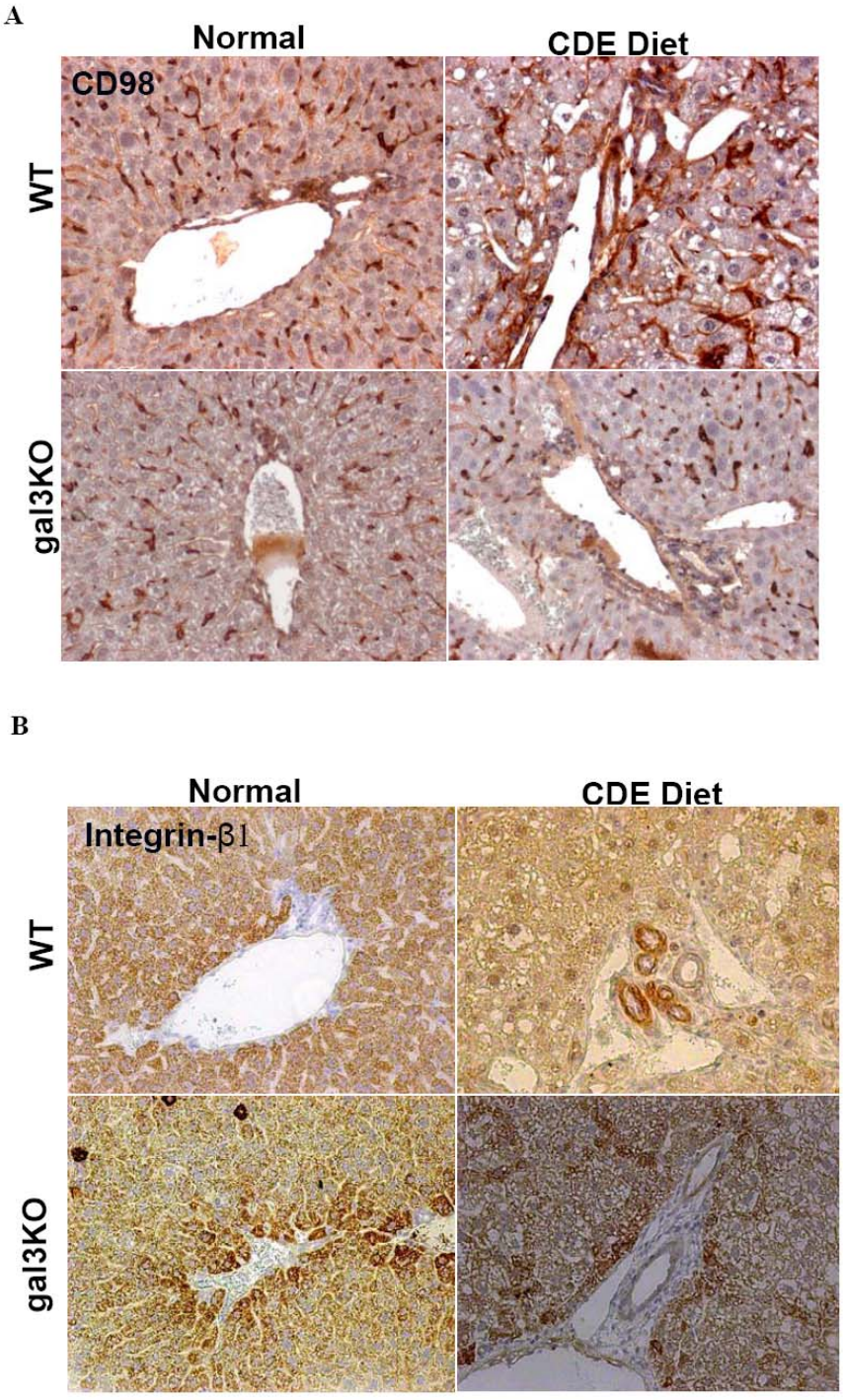

A
Normal
CDE Diet
CD98
WT
gal3KO
B
Normal
CDE Diet
Integrin-β1
WT
gal3KO

Figure 5.7 Different expression pattern of CD98 and integrin β1 in the livers of the CDE-fed WT and galectin-3 null mice. Immunostaining for (A) CD98 (B) integrin β1 in the livers of normal WT, galectin-3 null mice and CDE-fed WT and galectin-3 null mice. Original magnification: x 200.

5.3.7 Both CD98 and integrin β1 were expressed by PanCK or galectin-3 positive cells, especially on the dividing cells.

The expression of CD98, galectin-3 and integrin β1 on primary LPCs was then investigated. Anti-CD29 antibody was used to highlight integrin β1. CD98 was frequently expressed by panCK- positive LPCs. In particular, it was highly expressed on dividing panCK-positive LPCs (Fig. 5.8A, arrows). The co-expression of CD98 and galectin-3; galectin-3 and integrin β1; and CD98 and integrin β1 were all observed on primary LPC culture. (Fig 5.8A) Interestingly, CD98, galectin-3 and integrin β1 were expressed highly around the dividing cells. (Fig 5.8A, arrows)

A similar expression pattern of CD98, galectin-3 and integrin β1 was also observed in LPC line: BMOLs. CD98 and integrin β1 were both expressed by panCK- positive BMOLs. (Fig 5.8B) Similarly, the co-expression of CD98 and galectin-3 was also observed in BMOLs. (Fig 5.8B)

Figure 5.8

A

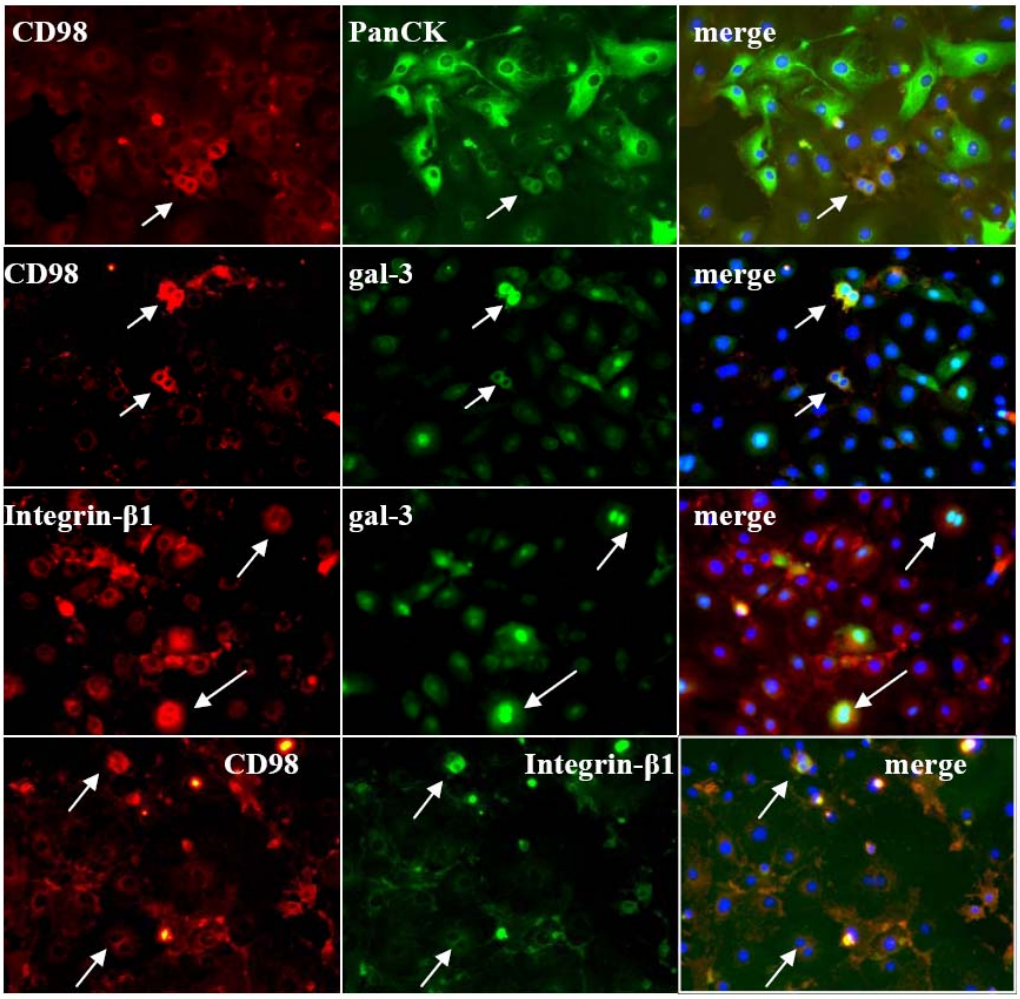

CD98
PanCK
merge
CD98
gal-3
merge
Integrin-β1
gal-3
merge
CD98
Integrin-β1
merge

Figure 5.8

B

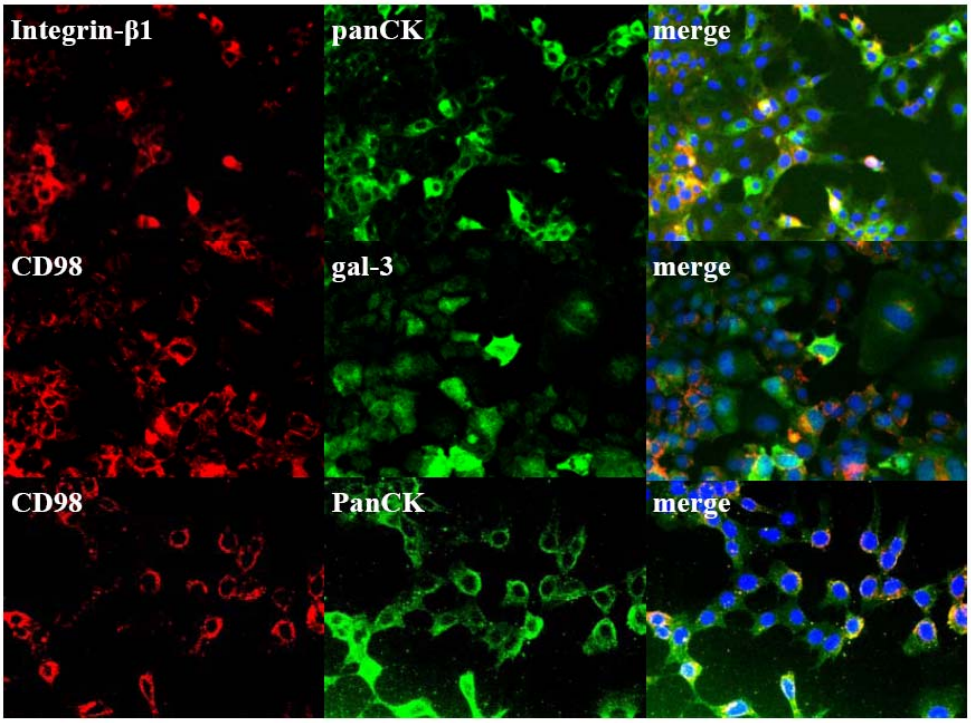


Figure 5.8 Expression of CD98,galectin-3, and integrin β1 on primary LPCs and LPC line: BMOLs (A)Double staining for panCK and CD98, CD98 and galectin-3, galectin-3 and integrin β1 CD98 and integrin β1 on primary LPCs. (B) Double staining for integrin β1 and panCK, CD98 and galectin-3, CD98 and panCK on LPC line: BMOLs. Original magnification: x 200.

5.4 Discussion

Previous studies have shown that galectin-3 plays a role in regulating cell cycle. Apart from activating cyclin D1 promoter (Lin et al., 2002), galectin-3 has also been demonstrated to regulate the expression of cell cycle inhibitors. Galectin-3 knockdown in human prostate cancer cells led to cell-cycle arrest at G1 phase, up-regulation of p21, and hypophosphorylation of the retinoblasma tumor supressor protein (pRb) (Wang. et al., 2009). This chapter demonstrated that cyclin D1 protein expression in galectin-3 null primary LPCs was significantly reduced compared to WT primary LPCs. In addition, the knock down of galectin-3 in the LPC line: BMOLs also significantly down-regulated cyclin D1 expression, confirming the role of galectin-3 in regulating cyclin D1 expression. Moreover, the expression of cell cycle inhibitors p16 and p21 was significantly up-regulated in galectin-3 null primary cells. This up-regulation of p16 and p21 was also observed in galectin-3 siRNA-transfected BMOLs. Surprisingly, after 5 days of transfection, the expression of p21 was reduced in the BMOL cells, regardless of whether they were transfected with scrambled or galectin-3 siRNA. This observation may be attributed to the fact that the cells were cultured for long period of time. However, the expression of p21 in the BMOL cells transfected with galectin-3 siRNA was still higher than that in the BMOLs transfected with scrambled siRNA. These findings suggest a role for galectin-3 in regulating the expression of cell cycle regulators. The absence or the knocking down of galectin-3 expression down-regulates cyclin D1 and up-regulates p16 and p21.

Galectin-3 has been shown to bind to β-catenin which is involved in the regulation of the Wnt signalling pathway, thus stimulates the gene expression of cyclin D1

(Shimura et al., 2004). Previous studies indicated that the down-regulation of galectin-3 reduces the level of β-catenin and cyclin D1 in human colon cancer cells (Song et al., 2009). Down-regulation of galectin-3 also resulted in the dephosphorylation of glycogen synthase kinase-3β (GSK-3β), which is involved in Wnt pathway (Song et al., 2009). However, this differential expression of β-catenin and GSK-3β reported by Song and co-workers was not observed in WT and galectin-3 null LPCs. Further research needs to be carried to investigate whether the Wnt pathway is involved in the mechanisms of galectin-3 regulating LPC proliferation.

How galectin-3 regulates cyclin D1 remains unknown. It has been shown that cell adhesion- mediated cyclin D1 promoter activation appears to occur through focal adhesion kinase (Zhao et al., 1998). However, it was also demonstrated that galectin-3 induces cyclin D1 promoter activity in human breast epithelial cells independent of cell adhesion. Lin et al (2002) proposed that galectin-3 might be involved in the enhancement/stabilization of nuclear protein-DNA complex formation at the CRE site of the cyclin D1 promoter (Lin et al., 2002). In this study it was observed that the phosphorylation of FAK and Akt were reduced when galectin-3 null LPCs were cultured on plastic plates. However, only a slight reduction of FAK phosphorylation was observed when galectin-3 null LPCs were cultured on laminin-coated plates. Unexpectedly, no difference in Akt phosphorylation was observed between WT and galectin-3 null LPCs when cultured on laminin-coated plates. This similar expression pattern was also observed when the expression of galectin-3 was reduced in BMOLs. Phosphorylation of FAK and Akt was reduced in the galectin-3 siRNA- transfected BMOLs cultured on plastic but not

on laminin-coated plates. This result suggests that other mediators may compensate for the loss of galectin-3 to mediate the phosphorylation of FAK and Akt when LPCs were cultured on laminin-coated plates. However, the phosphorylation of FAK and Akt normally occurs within 5 minutes to 1 hour. Therefore, the time points used in this study may not be suitable for the investigation of FAK and Akt phosphorylation. Further research need to be conducted to make any firm conclusion about this. Exogenous galectin-3 can be added into galectin-3 null primary LPCs at various time points from 5 minutes to 1 hour to further investigate whether galectin-3 mediate FAK and Akt phosphorylation when culturing LPCs on laminin-coated plates. Nevertheless, this study still demonstrated the role of galectin-3 in mediating the phosphorylation of FAK and Akt in LPCs when cultured on plastic plates.

Further research still needs to be conducted to investigate whether mediating cell-adhesion signalling pathways are involved in the mechanism by which galectin-3 regulates the expression of cell cycle regulators and LPC proliferation. It has been shown that basement membrane of bile ducts comprises laminin and type IV collagen (Terada et al., 1994; Yasoshima et al., 2000). However, whether integrin activation is involved in the regulation of LPC proliferation is not clear. It has been shown that both CD98 and galectin-3 interact with integrin β1 and can mediate certain integrin β1 functions (Fenczik et al., 1997; Merlin et al., 2001; Henderson et al., 2006; Feral et al., 2005; Hughes, et al., 2001). It is thus possible that galectin-3 regulates the binding activity of CD98 with integrin β1 which may mediate the adhesion of LPC to laminin and regulate LPC proliferation and differentiation. In this study, the preliminary data has shown that both CD98 and integrin β1 were expressed on panCK-positive LPCs *in vitro*. The co-expression of galectin-3 and CD98,

galectin-3 and integrin β1; CD98 and integrin β1 was observed in LPC culture. Interestingly, they were all expressed on dividing cells, suggesting that galectin-3, in conjuction with CD98 and integrin β1, may play an important role in the regulation of LPC proliferation.

Moreover, the expression of CD98 and integrin β1 is related to LPC activation. CD98 was expressed around panCK-positive LPCs. Also, CD98 expression was increased and especially localized around the area of LPC activation when LPCs were activated in the liver of CDE-fed WT mice, proposing that CD98 plays a role in regulating LPC activation. However, an increase in CD98 expression was not observed in the liver of CDE-fed galectin-3 null mice. This suggested that galectin-3 may regulate CD98 expression when LPCs were activated. Furthermore, integrin β1 was widely expressed on hepatocyte in the liver of normal WT mice. On the other hand, the expression of integrin β1 was restricted to the bile ductules in the liver of CDE-fed WT mice, suggesting that integrin β1 expression is associated with LPC activation. However, this expression pattern was not observed in galectin-3 null mice. Integrin β1 was widely expressed on hepatocyte both in the liver of normal and CDE-fed galectin-3 null mice. These results suggested a role for galectin-3 in modulating integrin β1 expression during LPC activation. Taken together, these data demonstrated that the expression of CD98 and integrin β1 were associated with LPC activation and may be regulated by galectin-3. However, further research needs to be conducted to assess whether galectin-3 and CD98 are both involved in integrin activation and the regulation of LPC proliferation.

In summary, this study indicated that the absence of or the knock down of galectin-3

expression down-regulated cyclin D1 but up-regulated p21 and p16 in LPCs. In addition, it seems that Wnt/β-catenin signalling pathway is not regulated by galectin-3 during LPC proliferation. Although the down-regulation of FAK and Akt phosphorylation was observed when galectin-3 null LPCs or galectin-3 siRNA-transfected BMOLs were cultured on plastic plates, more research needs to be done to confirm the role of galectin-3 in regulating FAK and Akt phophorylation during LPC proliferation when culturing LPCs on laminin. Nevertheless, these results still suggest that galectin-3 may regulate LPC proliferation dependent on the cell-adhesion signalling pathway. Furthermore, the expression of CD98 and integrin β1 was associated with LPC activation and regulated by galectin-3. In addition, CD98 and integrin β1 were both expressed on PanCK positive or galectin-3 positive cells, especially on dividing cells, which further suggested that CD98 and integrin β1 may be involved in regulating LPC proliferation. Further research is needed to assess whether galectin-3 in synergy with CD98, activates integrin signalling and regulates LPC proliferation.

CHAPTER 6

CONCLUDING REMARKS AND POTENTIAL FUTURE STUDIES

In this study, the role of galectin-3 in regulating LPC proliferation and differentiation was investigated. I indicated that galectin-3 expression is strongly associated with LPC reaction. The essential role of galectin-3 in initiating LPC activation and regulating LPC proliferation are demonstrated here by assessing the mouse model of LPC induction, the CDE diet. In addition, I demonstrated that galectin-3 is required for LPC proliferation and maintaining LPCs in an undifferentiated state on laminin, either by investigating primary galectin-3 null primary LPCs or galectin-3 siRNA transfected BMOLs. Moreover, I have also shown that the extracellular binding activity of galectin-3 is important for LPC adhesion to laminin and LPC proliferation *in vitro*. Furthermore, I indicated that the absence of galectin-3 or the knock down of galectin-3 expression down-regulates cyclin D1 but up-regulates p21 and p16. The adhesion-mediated signalling pathway such as phosphorylation of FAK and Akt may also be involved in this mechanism. Finally, the hypothesis that integrin β1 and CD98 are involved in the regulation of LPC behaviour is also proposed.

Laminin has been suggested to play an important role in constituting the LPC niche and maintaining LPCs in an undifferentiated state within the niche (Lorenzini et al., 2010). This study confirmed that laminin can promote LPC proliferation and enhance LPC adhesion. In addition, LPCs differentiated into hepatocyte and biliary cells more rapidly on plastic plates under conditions which promote differentiation, compared to culturing on laminin-coated plates. These data confirm that laminin is essential for

LPC proliferation and maintaining LPCs in a less differentiated state.

In vivo, no obvious LPC induction and significantly reduced LPC proliferation was observed in the livers of CDE-fed galectin-3 null mice compared to WT controls. In addition, galectin-3 was essential in LPC proliferation but has a negative effect on LPC differentiation. The absence of galectin-3 leads LPCs to differentiate more rapidly on laminin, suggesting that galectin-3 is required to maintain LPCs in an undifferentiated state on laminin. These findings also suggested that galectin-3 may be crucial in mediating the homeostatic balance between proliferation and differentiation of LPCs. For future research, the LPC lineage tracing mice tools can be used to investigate whether galectin-3 null mice have a more rapid differentiation of LPCs into hepatocytes and biliary cells, compared to the WT mice *in vivo*. Moreover, the *in vivo* rescue experiment, introducing an adenovirus vector expressing galectin-3 (Adv) back to the galectin-3 null mice can also be conducted to investigate whether LPC induction can be achieved in the livers of galectin-3 null mice by exogenous galectin-3. Furthermore, both WT and galectin-3 null primary LPCs can be transplanted into WT mice to investigate whether galectin-3 is important for LPCs to repopulate the liver during liver injury. At the converse, WT LPCs can be transplanted into both WT and galectin-3 null mice to investigate whether the presence of galectin-3 in the supporting environment of the LPC niche is crucial for LPC to repopulate the liver during injury.

The apparent co-localisation between galectin-3 expressing cells and the supporting cells within the LPC niche: macrophages, was demonstrated. This suggested that galectin-3 may itself be involved in cross-talk between LPC and their supporting

environment. Galectin-3 may be important in supporting laminin to retain the LPCs within the niche for expansion. In this study, galectin-3 null mice fail to form a laminin sheaf around LPCs and have less recruitment of macrophages to LPCs, suggesting that galectin-3 may play a crucial role in regulating niche formation. Galectin-3 is normally strongly expressed by macrophages. Thus the presence of galectin-3 in macrophages, one of the supporting cells in the LPC niche, may also play an important role in regulating LPC behaviour. Further studies such as *in vitro* co-culture of galectin-3 null macrophages with WT LPCs still need to be conducted to analyze whether the absence of galectin-3 in macrophages has an effect on LPC proliferation and differentiation.

The importance of galectin-3 in regulating LPC proliferation and differentiation was demonstrated here. However the mechanisms by which galectin-3 regulate LPC proliferation and differentiation has not been completely investigated. This mechanism may be complicated due to the wide distribution of galectin-3 at the cell surface, extracellularly and intracellularly. There are two possibilities: (a) extracellular galectin-3 may regulate LPC proliferation by mediating LPC adhesion to laminin. The essential role of the extracellular binding activity of galectin-3 in LPC proliferation and LPC adhesion to laminin was described in this study. Lactose, which can block the extracellular binding activity of galectin-3, inhibited LPC adhesion to laminin and the growth of LPCs on laminin. (b) Galectin-3 may modulate LPC proliferation by regulating the expression of cell cycle regulators. In this study, significant down-regulation of cyclin D1 and up-regulation of p16 and p21 was observed both in galectin-3 null primary cells and galectin-3 siRNA transfected BMOLs.

Galectin-3 may regulate cyclin D1 expression by regulating the cell-adhesion mediated signal pathway or by mediating the enhancement/stabilization of nuclear protein-DNA complex formation at the CRE site of the cyclin D1 promoter to stimulate cyclin D1 gene expression (Lin et al., 2002). It has been shown that cell adhesion signalling is a pre-requisite for growth-induced cyclin D1 expression (Le Gall et al., 1998). In addition, the transcription of cyclin D1 is down-regulated following the loss of cell adhesion (Zhu et al., 1996). However, it has also been demonstrated that galectin-3 induces cyclin D1 promoter activity in human breast epithelial cells independent of cell adhesion (Lin et al., 2002). The exact mechanism of galectin-3 regulating cyclin D1 expression during LPC proliferation still needs to be investigated. Nuclear galectin-3 has been shown to bind to β-catenin which is involved in the regulation of Wnt signalling pathway to localize β-catenin to the nucleus and stimulate cyclin D1 expression (Shimura et al., 2004). However, the down-regulation of β-catenin activation was not observed in galectin-3 null LPCs. Further research need to be carried to analyze whether galectin-3 is involved in β-catenin activation during LPC proliferation. For example, luciferase reporter plasmid containing β-catenin binding sites can be transfected into WT and galectin-3 nuill LPCs to examine the β-catenin binding activity.

Whether galectin-3 regulates cyclin D1 gene transcription through binding to other nuclear protein-DNA complexs remain unclear. Although the down-regulation of phosphorylation of FAK and Akt was found in primary galectin-3 null LPCs and galectin-3 siRNA transfected BMOLs, this effect was not obvious when the cells were cultured on laminin-coated plates. This result may due to the long term culture of 4 days since the phosphorylation of FAK and Akt normally occur within 5 min to

1 hour. Whether galectin-3 plays an important role in regulating the phosphorylation of FAK and Akt during the LPC proliferation on laminin was not completely investigated. The recombinant galectin-3 can be introduced to galectin-3 null LPCs extracellularly to analyze their short term effects on phosphorylation of FAK and Akt. Moreover, whether galectin-3 regulates cyclin D1 expression is dependent on cell adhesion remains unknown. For future research, cyclin D1 expression or cyclin D1 promoter activity of galectin-3 null LPCs which are cultured in suspension can be assessed by immunobloting or luciferase assay to examine whether cell adhesion is required for regulating cyclin D1 expression. Furthermore, the location of galectin-3 in proliferating and quiescent LPCs can be examined in future studies since the localization of galectin-3 in the cells may be coordinated with the proliferation state.

Finally, CD98 and integrin β1 may also be involved in the regulation of LPC behaviour by galectin-3. Galectin-3 has been shown as an endogenous cross-linker of CD98 which ligates the glycosylated extracellular domain of CD98, mediating integrin clustering on the surface of cells to increase avidity of binding (Hughes et al., 2001). This mechanism may mediate cell adhesion to laminin. Whether galectin-3 can trigger CD98 mediated-integrin activation remains unclear. This study has shown some preliminary data demonstrating that the expression of CD98 and integrin β1 were both strongly associated with LPC induction and may be regulated by galectin-3. In addition, both CD98 and integrin β1 were expressed on PanCK positive LPCs *in vitro*, especially on proliferating cells. The co-expression of galectin-3 and CD98, galectin-3 and integrin β1; CD98 and integrin β1 was also observed in LPC culture. Whether CD98 and integrin β1 regulates LPC induction, proliferation and differentiation still needs to be investigated. The conditional CD98

null mice and integrin β1 deficient mice can be supplemented with CDE diet to assess their roles in LPC induction and proliferation *in vivo*. In addition, BMOLs can be transfected with CD98 siRNA or treated with integrin β1 blocking antibody to examine their roles in LPC proliferation and differentiation *in vitro*. Moreover, the interactions between CD98 and galectin-3 can be analyzed by immunoprecipitation to investigate the interaction between galectin-3 and CD98 of LPCs.

In summary, there are three major findings in my study: (1) Galectin-3 is required to maintain LPCs in an undifferentiated state on laminin. (2) Blocking the extracellular binding activity of galectin-3 contributes to reduced LPC adhesion to laminin. (3) The absence of or the knock-down of galectin-3 expression leads to a cell cycle arrest in LPCs. These data proposed that galectin-3 is a key signalling intermediary in the LPC niche, regulating homeostatic balance between proliferation and differentiation of LPC, thus controlling regeneration.

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